Vol. 50, 1973

Acta Haematologica

International Journal of Haematology
Journal International d'Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology

S. Karger · Basel · München · Paris · London · New York · Sydney

S. Karger Basel München Paris London New York Sydney
Arnold Böcklin-Strasse 25, CH-4011 Basel (Switzerland)

Printed in Switzerland by Buchdruckerei National-Zeitung AG, Basel

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ly tested with success in acute lymphoblastic leukaemia (ALL) Out of 30 patients treated with 2 mg m² of VCR weekly and 60 mg m² of Pred daily 9 (30%) achieved a complete or partial remission and 6 of the 9 had a complete haematologic remission with return of the marrow morphology to normocellularity or to the granulocytic hyperplasia characteristic of the myelocytic phase of CGL In addition in 5 of these patients the aneuploid line that characterized their blastic transformation completely disappeared with a return to the chromosomal constitution of the chronic phase of the disease (46 chromosomes Ph¹ positive) The mean duration of the first remission in the complete remitters was 5 months Furthermore in one patient having presented with the clinical and haematologic features of acute myeloblastic leukaemia but with an aneuploid Ph¹ positive blast cell population a prolonged remission was obtained resulting in a 28 month survival with the features of CGL, while progressive refractoriness to chemotherapy was associated with the reappearance of the same aneuploid blast cell line and by the emergence of an additional aneuploid clone [7]

Since we too have been using much the same combination for the last three years we should like to report our own experiences which besides confirming the efficacy of this simple treatment have enabled us to observe a marked difference between the two fundamental cytologic types of metamorphosis

Clinical Observations

A VCR-corticosteroid (generally dexamethasone or prednisolone) combination practically superimposable to the schedule of CANELLOS *et al* [6] has been employed for the treatment of metamorphosis of CGL in our Department for the last 3 years However no single dose of VCR superior to 2 0 mg was ever given in order to avoid severe neurotoxicity

The diagnosis of metamorphosis was made according to the generally accepted criteria [4-6 12 19 22 25] however a distinction was made between genuine *blastic transformation* of bone marrow and blood (fig 1) composed of basophilic agranular undifferentiated cells corresponding to the so-called promyeloblastic type of MATHÉ *et al* [19] and what we prefer to call the granular type of metamorphosis that is a generally gradual (more rarely abrupt) augmentation of immature granulocytic cells always characterized by the abundance of azurophilic granules in the cytoplasm when stained with the Romanovsky s mixtures

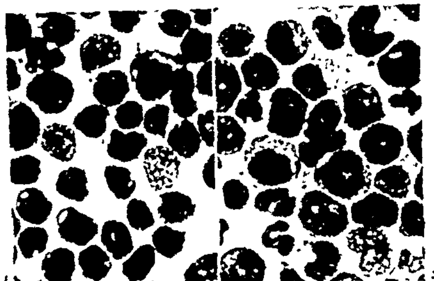


Fig. 1. Typical agranular blastic terminal metamorphosis of CGL.
Fig. 2. The appearance of granular metamorphosis, which is different both from the former type and from the picture of florid uncomplicated CGL.

(fig. 2). At times the number and uniform appearance of such cells mimicked the appearance of acute promyelocytic leukaemia (APL), as it has been already remarked [4]; however, in our experience, Auer rods are abundant in APL, but always absent in metamorphosis. In this category, 2 cases with marked hypereosinophilia (>50%) were found, although eosinophilia had not been prominent all along the myelocytic phase.

Although mixed pictures could be observed in a few cases, both for the sake of simplicity and in order to obtain a better evaluation of the therapeutic results, all cases were divided in 2 categories according to the predominant feature, that is an 'agranular' and a 'granular' type. Both were treated with the VCR steroid combination for periods never exceeding 6 weeks.

The results are shown in table I, in which the well-known criteria for the acute leukaemias have been adopted for the evaluation of complete and incomplete remissions.

Complete and incomplete remissions were subsequently treated either with a combination of 6-MP (2.5 mg/kg/day) and MTX (15 mg per

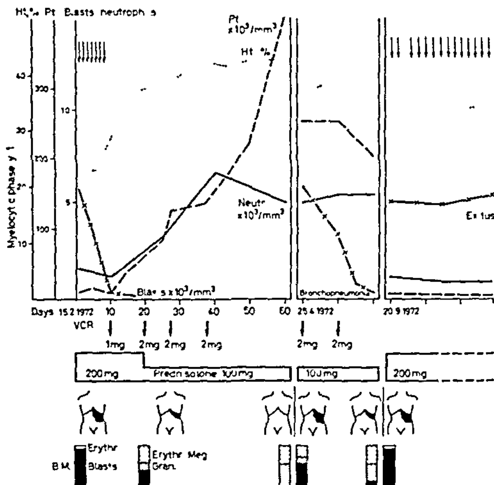


Fig 5 Non granular blastic metamorphosis of CGL

ized CANELLOS *et al* [6] have presented evidence indicating that the hypodiploid cell lines are significantly more sensitive to this combination, and have speculated that they may contain less microtubular spindle protein, and be accordingly more sensitive to the antimicrotubular properties of VCR However reduction of chromatin material as in hypodiploidy does not appear to be necessarily coupled with a diminution of microtubular protein which should be ascertained separately In addition the prompt and dramatic oncolytic effect of corticosteroids on these cells cannot be explained in terms of microtubular damage steroids notoriously attack lymphoblasts also in the resting stage, besides arresting them in G_1 [11] a mechanism which has been recently

shown to be operative also for VCR [21]. It is conceivable that the sensitivity of these cells may result from a combination of their proliferative state and intrinsic metabolic properties.

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Acta Haemat. 50: 9-18 (1973)

A Comparison of the Three *in vivo* Assays for Haemopoietic Stem Cells

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Abstract. The effect of a proliferation independent (nitrogen mustard) and 4 proliferation-dependent (methotrexate, vinblastine, cyclophosphamide and 5-fluorouracil) cytotoxic agents has been investigated concurrently on the 3 *in vivo* assays (colony forming units, erythroid repopulating ability and granulocytic repopulating ability) for haemopoietic stem cells. These preliminary results show that the proliferation dependent agents produce different responses in the 3 assays consistent with the presence of 3 different cell populations proliferating at different rates. The findings tend to support published hypotheses on the existence of 3 different stem cell populations (possibly 'committed' stem cells).

Key Words
Cytotoxic agents
Erythroid repopulation
Granulocytic repopulation
Haemopoiesis
Proliferation rates
Spleen colony formation

Several *in vivo* assays have been used to estimate the proliferation of haemopoietic stem cells. The response to erythropoietin in polycythaemic mice [10] is now known to measure a cell population (the erythropoietin responsive cells) distinct from the haemopoietic stem cells [3, 4, 18, 23]. However, there are 3 other assays which are still considered to measure stem cells. The spleen colony technique [22], the erythroid repopulating ability technique, based on the ability of transplanted bone marrow to restore erythropoiesis, as measured by ^{59}Fe uptake, in bone marrow-depleted recipients [15, 24] and the granulocytic repopulating ability technique in which the granulopoietic capacity of transplanted bone marrow is measured by the response of the peripheral blood neutrophils to endotoxin [5, 12].

If the concept of a single type of bone marrow 'stem' cell, which is multipotential (i.e. can produce differentiated progeny of all haemopoietic

cell lines) is valid, it would be expected that for a given pre-treatment to the donor animal, the same result would be obtained from each of the 3 assays, provided that the treatment did not influence the differentiation pathways of the surviving multipotential 'stem' cells. The evidence so far available, however, indicates that the erythroid and granulocytic repopulating abilities, in mice, respond in different ways to X-irradiation and cyclophosphamide [13, 14] and, in rats, to cyclophosphamide and repeated injections of methotrexate [7, 8].

It was felt that the best way of investigating the relationship between these precursor cell assays was to measure all three, *concurrently*, using the same sample of bone marrow. The donor mice were treated with single doses of various cytotoxic drugs taken from each group of the classification scheme for cytotoxic agents, as established by BRUCE *et al.* [5] on the basis of target cell proliferation rates. Class I agents (as exemplified by nitrogen mustard) show exponential dose/response graphs with both slowly and rapidly proliferating cells having a similar sensitivity. Class II agents (phase specific, e.g., methotrexate and vinblastine) act over one phase of the cell cycle. The dose/response graphs show plateaus because only those cells in the sensitive phase of the cycle are killed. The plateau is at a lower survival for rapidly proliferating cells than for slowly proliferating cells, since more cells pass through the sensitive phase during the time for which the drug is active. Class III agents (cycle-specific, e.g., cyclophosphamide and 5 fluorouracil) act on proliferating cells throughout the cell cycle. The dose response graphs are exponential but the graph for rapidly proliferating cells is steeper than for slowly proliferating cells.

The following results represent our preliminary findings in which the response in the 3 *in vivo* assays for haemopoietic stem cells have been compared. Nitrogen mustard (HN2), methotrexate (MTX) and cyclophosphamide (CY) were studied in some detail. Pilot experiments were carried out using vinblastine (VB) and 5-fluorouracil (5-FU) to determine whether the responses observed after MTX and CY were specific for MTX and CY or were characteristic of class II (MTX and VB) and class III (CY and 5-FU) agents generally.

Materials and Methods

Chemicals. Nitrogen mustard (mustine hydrochloride) was obtained from Boots Pure Drug Co. Ltd., Nottingham; methotrexate from Cyanamide International, Pearl River, N.Y.; vinblastine sulphate (Velbe) from Eli Lilly & Co. Ltd., Basing

stoke, cyclophosphamide ('Endoxana') from Ward Blenkinsop & Co. Ltd., London, and 5 fluorouracil from Roche Products Ltd., Welwyn Garden City.

All these agents were dissolved in saline and the solutions prepared immediately before injection. The chemicals were administered intravenously, to the donor mice 24 h before the marrow was taken for assay.

Animals. C57Bl ♂ mice, aged approximately 12 weeks at the start of experimentation, were used throughout. Between 3 and 12 mice served as bone marrow cell donors in each group. The bone marrow from these animals was assayed simultaneously for colony forming units, erythroid and granulocytic repopulating abilities.

Approximately 4 h before receiving the bone marrow, the recipients for all 3 assays were exposed to 700 rad ^{60}Co-γ-rays (dose rate 45 rad/min). This dose of irradiation was sufficient to reduce the mean endogenous colonies to less than one per spleen.

At least 6 recipients per group were used for the spleen colony and erythroid repopulating ability assays and 7 for the granulocytic repopulating ability assay.

Varying numbers of treated bone marrow cells were transplanted such that the responses in all 3 assays were always on the linear part of the respective dose response graphs.

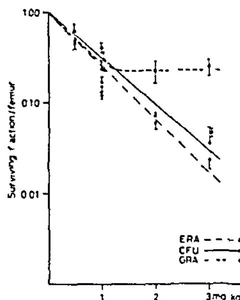
Spleen colonies (CFU). The method for the assay of colony forming units (CFU) was basically that described by TILL and MCCULLOCH [22]. Eight days after bone marrow transplantation, the recipient animals were killed and their spleens excised and fixed in Bouin's solution. All macroscopic colonies on the flat surface of the spleens were counted.

Erythroid repopulating ability (ERA). This technique has been fully described earlier [15, 24]. ^{59}Fe was injected into the recipient mice 7 days after bone marrow transplantation and the uptake into the blood determined 24 h later. Blood volume data were used to express isotope uptake in terms of total activity in the blood animal according to the previously reported method [24].

Granulocytic repopulating ability (GRA). The peripheral blood granulocyte response to endotoxin was assayed 9 days after bone marrow transplantation by a modification of the method of HELLMAN and GRATE [12]. 15 μg *S. typhosa* endotoxin (Difco Chemical Co.) were injected intraperitoneally into each recipient animal and 8 h later, at the time of maximum granulocyte response [21], blood samples were taken and the concentration of granulocytes in the blood determined from total leukocyte counts (measured with haemocytometers) and peripheral blood leukocyte differentials determined from blood smears.

HELLMAN *et al.* [13] have shown that the GRA of irradiated mouse bone marrow is not significantly different when determined 9 or 11 days after transplantation and, in rats, the GRA of marrow treated with cytotoxic drugs is independent of the time after transplantation [6]. Therefore, it was considered that a 9-day assay of GRA would be suitable for these preliminary investigations.

Calculation of results. In all 3 assays, the results are expressed relative to one control. The mean and standard errors were calculated from the individual results in each group of recipients. The means ± standard errors of the experimental groups are expressed as fractions of the relevant control value ± its standard error. Thus, the points on the following figures represent the surviving fraction of control ± an error which includes the variation in the control group.



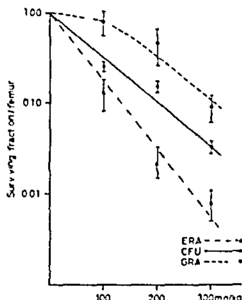


Fig 1 Erythroid repopulating ability (ERA) colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur of mouse bone marrow 24 h after nitrogen mustard Vertical bars indicate ± one standard of the mean For clarity, the top or bottom error bars have been omitted from certain points Mean results of 3 experiments

Fig 2 Erythroid repopulating ability (ERA), colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur of mouse bone marrow 24 h after cyclophosphamide Vertical bars indicate ± one standard error of the mean Mean results of 3 experiments

Results

Nitrogen mustard had similar effects on the ERA and CFU both decreasing exponentially with dose as expected for a class I agent However, the GRA decreased to a plateau at the level of approximately 20% survival (fig 1) Since after irradiation (another class I agent), it has been shown [12] that all 3 assays show an exponential decline with increasing dose, the plateau in GRA after nitrogen mustard would seem specific to this agent rather than class I agents generally

Single doses of *methotrexate* produced only approximately 50% depression in survival for all 3 assays All doses used appeared to be on the plateaus in the dose response curves and there was possibly a slightly greater survival of GRA than of ERA and CFU (table I)

Table I

Dose of methotrexate mg/kg	Surviving fractions/femur of		
	ERA	CFU	GRA
40	0.55 ± 0.06	0.51 ± 0.04	0.84 ± 0.23
100	0.65 ± 0.10	0.47 ± 0.06	0.83 ± 0.23
200	0.65 ± 0.18	0.59 ± 0.04	0.72 ± 0.27

Erythroid repopulating ability (ERA), colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur (± one standard error of the mean) 24 h after methotrexate. Mean results of 3 separate experiments.

Table II

Dose of vinblastine mg/kg	Surviving fractions/femur of		
	ERA	CFU	GRA
0.5	0.04 ± 0.01	0.10 ± 0.01	0.24 ± 0.07
1.0	0.05 ± 0.01	0.04 ± 0.01	0.12 ± 0.02

Erythroid repopulating ability (ERA), colony forming units (CFU), and granulocytic repopulating ability (GRA) per femur (± one standard error of the mean) 24 h after vinblastine sulphate. Mean results of 2 experiments.

The results with *vinblastine* (table II) were essentially similar to those obtained following methotrexate (table I). In view of the findings of SILINI *et al.* [20] it was not possible to be certain that the doses used corresponded to the plateau regions on the dose response curves as demonstrated for CFU by BRUCE *et al.* [5] using similar doses.

The survival in all 3 assays after *cyclophosphamide* decreased exponentially with increasing dose but with different slopes for each assay (fig. 2). There was some indication of an initial shoulder on the GRA curve. The CFU curve lay between those obtained for GRA and ERA. Using similar doses, HELLMAN *et al.* [14] observed a reduction in GRA to a plateau level of 30% survival following cyclophosphamide which contrasts with the exponential decrease shown in figure 2. A direct compari-

Table III

Dose of 5 FU mg/kg	Surviving fractions femur of ERA	CFU	GRA
40 0	0 14 ± 0 02	0 19 ± 0 02	0 62 ± 0 14
80 0	0 11 ± 0 002	0 09 ± 0 01	0 19 ± 0 05
100 0	0 009 ± 0 003	0 06 ± 0 01	0 09 ± 0 01

Erythroid repopulating ability (ERA) colony forming units (CFU) and granulocytic repopulating ability (GRA) per femur (± one standard error of the mean) 24 h after 5 fluorouracil Mean results of 3 experiments

son between the results of Hellman *et al* [14] and those now reported is difficult, since in Hellman's study cyclophosphamide produced only a small depression of CFU Other reports [5, 26] have shown cyclophosphamide to have a marked effect on CFU similar to that now reported

Preliminary results with *5-fluorouracil* (table III) indicated that the type of response was similar to that seen after cyclophosphamide (fig 2) and suggest that the different responses in these 3 assays were typical of class III agents

Discussion

It has been shown that erythroid and granulocytic repopulating abilities and spleen colony-forming units each respond in different ways to treatment with certain cytotoxic agents The reduction of granulocytic repopulating ability to a plateau following nitrogen mustard (fig 1) was not found with the other 2 assays The cycle-specific agents, cyclophosphamide (fig 3) and 5 fluorouracil (table III) produced exponential decreases in survival but with different slopes for the 3 assays and there appeared to be initial shoulders on the GRA curves The results also suggested that the phase-specific agents methotrexate (table I) and vinblastine (table II) had less effect on GRA than on CFU and ERA

Both the CFU assay [9] and the cells measured with the repopulating assays [3, 25] measure populations of primitive haemopoietic cells with considerable capacity for proliferation and which have distinct kinetic characteristics from the more mature, morphologically recognisable, haemopoietic cells All 3 *in vivo* stem cell assays are dependent, probably to

varying degrees, on the proliferative capacity of the viable, transplanted stem cells. This ability may or may not be altered by treatment of the donors with cytotoxic agents, although it would depend on an induced defect in differentiation and/or maturation being transmitted through many cell divisions [9]. Should a change in the output of mature cells/stem cell occur, it might be expected to influence the CFU and repopulating assays in different ways. Since the CFU assay depends on the scoring of clones of the progeny of individual stem cells in one organ only (i.e. the spleen), changes in cell output from the stem cells could possibly result in a reduction in colony size without, necessarily, in colony number. Changes in cell production, however, might contribute to changes in the repopulating assays which depend on the integrated output of mature differentiated cells derived from all the transplanted repopulating cells. If all 4 proliferation-dependent cytotoxic agents (MTX, VB, CY and 5-FU) produced a similar block in differentiation and/or maturation specific to the same haematological cell line, then the different survival curves, reported above, might be expected.

The type of mature cell produced may depend on the organ (e.g. bone marrow or spleen) in which the transplanted stem cell lodges and proliferates [27]. It seems unlikely, however, that drug treatment of the donors would lead to a redistribution of surviving stem cells in the recipients sufficient to account for the different responses of the 3 assays.

The CFU, ERA and GRA assays may measure distinct cell populations which respond in different ways to treatment with proliferation dependent cytotoxic agents. With the exception of the GRA curve after nitrogen mustard (see above), the shapes of the dose response curves for the 3 assays are as expected for three different cell populations proliferating at different rates [5]. These results, therefore, favour the existence of different populations of stem cells, and would be consistent with the considerable amount of evidence from other types of investigation [1, 2, 7, 8, 17, 18] which have suggested the existence of different populations of repopulating cells committed to separate lines of haemopoietic differentiation. The present studies with proliferation-dependent cytotoxic agents then lead to the conclusion that the erythroid repopulating cells proliferate rather more quickly, and the granulocytic repopulating cells somewhat unexpectedly, more slowly than the CFU. Results with the *in vitro* tritiated thymidine suicide technique[1] in a different strain of mice to those

[1] Dixon, C. D. R. and Constable, T. B., unpublished observations.

used in the present report suggest similar differences in the proliferation rate of the cells measured with these 3 assays

Further studies need to investigate the response in the CFU, ERA and GRA compartments with time after administration of the cytotoxic agents and to determine any alteration in the seeding efficiency of the CFU or in colony size Also a comparison of the responses in the 3 *in vivo* assays for stem cells, particularly the granulocytic repopulating ability, with those obtained using the *in vitro* bone marrow cloning technique will be of considerable interest since the granulocytic repopulating cells appear to be proliferating more slowly than the CFU while the available evidence [11, 16 19] suggests that the cells producing granulocytic colonies *in vitro* are proliferating more rapidly than the CFU

Acknowledgement The authors thank Miss W WOOLLISCROFT for her excellent technical assistance during the course of the experiments reported in this paper

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Acta Haemat. 50: 19–24 (1973)

Granulocyte Alkaline Phosphatase Activity: A Measure of the Emergence Time of Mature Marrow Neutrophils?

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Abstract. Early release of mature bone marrow granulocytes either with 'empty' stores or with exaggerated turnover rate of the marrow store furnishes more alkaline phosphatase positive neutrophils, whereas release of already stored cells furnishes more alkaline phosphatase negative cells. Thus, providing other factors, such as release of splenic or marginal granulocytes, do not disturb the condition, neutrophil alkaline phosphatase activity appears to reflect the bone marrow retention of candidate cells. The biphasic response to adrenal steroids could be explained by this hypothesis.

Key Words
Agranulocytosis
Bone marrow aplasia
Cell release from bone marrow
Granulocytopoiesis
Leukocyte alkaline phosphatase
Leukocyte kinetics
Pancytopenia

A relatively abundant amount of works deals with the explanation of granulocyte alkaline phosphatase positivity and negativity. Owing to the fact that, in general, enzyme activity diminishes with cell age, the decisive role of cell life span was considered [1], but it was subsequently suggested that granulocytes released from the bone marrow, either as a result of some injury, or of external stimulation, such as endotoxin or ethiocholanolone, are alkaline phosphatase negative [2–4] and positivity develops subsequently in the vascular bed. In this sense, a shift to alkaline phosphatase negative cells would mean something like reticulocytosis. Quite recently, Pederson and Hayhoe [5] theoretized that low granulocyte alkaline phosphatase activity in chronic granulocytic leukaemia (CGL) is a manifestation of incomplete maturation of cells, and the rise of activities in so-called blastic crises of CGL might be caused by an influx of fully matured granulocytes whose origin is the spleen rather than the bone marrow. Interestingly, a rapid transit through the marrow ma-

turation compartment was mentioned as a possible explanation for the low enzyme activity of CGL granulocytes [1]

I now report on 4 cases of drug induced granulocytopenia in which the alkaline phosphatase activity of granulocytes was investigated, both in bone marrow and peripheral blood smears, at the earliest phase of recovery of granulocytopoiesis Cases 1 and 2 suffered from pancytopenia, whereas cases 3 and 4 had overwhelming granulocytopenia Five cases of chronic idiopathic pancytopenia with hypocellular marrow (controlled by trephine biopsy) have been included, too

Method

The modified Kaplow method described by HAYHOE and QUAGLINO [6] was applied to demonstrate alkaline phosphatase and activities were scored, accordingly, from 0 to 4 The upper score limit regarded as non pathological in our laboratory, is 150

Case Histories

Case 1 A 57 year old man with marginal erythrocytosis has been erroneously diagnosed as suffering from polycythaemia vera in a hospital and ingested daily 750 mg dibromomannitol (DBM) for 3 weeks (?) without adequate blood cell control He was admitted to the clinic 18 days after the withdrawal of DBM He suffered from septicaemia, his granulocyte count was zero, and only a few hundred small lymphocytes were present in peripheral blood smears The first bone marrow samples were severely hypocellular contained a few cell types only small, and occasional medium sized lymphocytes plasma cells, macrophages and a somewhat homogenous early progenitor cell population

The first granulocytes (200 μl) appeared in the circulating blood as soon as 2 days after the above mentioned bone marrow investigation – which disclosed virtual absence of recognizable granulocyte precursors (1/1 000 cells) – indicating an extremely rapid whole marrow transition time in this emergency situation and suggesting immediate release from empty reservoirs [2] Along with this the representations of the potentially alkaline phosphatase positive granulocytes increased from 1/1 000 cells to 50 1 000 cells during a 72 hour period At this time practically all bands and segmented neutrophils showed remarkable alkaline phosphatase positivity (table I) However at least 2 factors influenced this result sepsis and prednisolone administration Two years after recovery, the patient is free of any haematological pathology

[1] Many other workers have made significant contributions to this problem but no attempt was made to review the literature

[2] Otherwise some atypical origin of these granulocytes should be presumed

Table I Intensity of granulocyte alkaline phosphatase (scores from 0 to 4) in % early at the recovery of injured granulocytopoiesis

Case No	Circulating blood 0	1	2	3	4	Bone marrow 0	1	2	3	4
1	–	20	30	35	12	6	31	38	18	7
2[1]	–	–	2	10	88	–	–	–	7/10	3/10
3	–	–	5	41	54	–	–	15	24	61
4[2]	5	3	8	43	41	6	7	25	48	14

[1] Only 50 peripheral granulocytes were calculated in this case and 10 mature granulocytes could be discovered in the marrow

[2] Metamyelocytes, or even myelocytes were positive in the marrow

Case 2. A 59 year-old woman, another patient treated by dibromomannitol on the base of unproven polycythaemia vera ingested 6.5–10.0 (?) g of the drug during a 9-day period and was admitted to the clinic in a poor condition with 200 WBC/µl 23 days after withdrawal of DBM. The bone marrow exhibited predominant fatty areas along with groups of mainly lymphocytes and plasma cells, but occasional progranulocytes were present as well as mature eosinophils. Mature neutrophils were, however, virtually absent.

Although clinical sepsis was not overwhelming, the patient was placed in a pathogen-free room, got wide-spectrum antibiotics, but prednisolone was not given. Table I shows the phosphatase scores at the day of the death of the patient, the 2nd day of our observation. She had hypertensive cardio-vascular disease with congestive splenomegaly and apoplexy terminated her life.

Case 3. A 64 year-old diabetic woman had severe agranulocytosis after about 5 weeks of treatment by the oral antidiabetic drug Invenol®. Her WBC count was 250/µl and her bone marrow differential was quite similar to that of case 2. Withdrawing the harmful drug, she became free of fever in the pathogen-free room already 2 days before the bone marrow investigation, and did not receive prednisolone at this period. The peripheral WBC count rose to 3000/µl within 2 days, 25% of them being granulocytes. Phosphatase values at this early period of recovery, i.e. the 2nd day of sudden clinical and haematological improvement are represented on table I.

Case 4. A 72 year-old man with Kaposi's angiosarcomatous developed a clinically not severe granulocytopenia caused by vincristine and cyclophosphamide therapy. The circulating granulocyte count dropped to 470/µl with about 200 neutrophils, but there was no fever and/or recognized systemic infection, the bone marrow cellularity was only moderately reduced, and every member of the granulocytopoietic series was present with a shift to the left. Prednisolone was not given, even before. At the earliest phase of recognized improvement of granulocyte counts (870/µl), i.e. at the beginning of an apparently spontaneous recovery of

granulocytopoiesis without any therapeutic measure alkaline phosphatase activities were investigated (table I)

Cases 5 9 Our last 5 cases of true chronic idiopathic aplastic pancytopenia exhibiting severe bone marrow hypocellularity as controlled by trephine biopsy, showed granulocyte alkaline phosphatase scores over 250 in peripheral smears, before any recent therapy and in the absence of clinically recognizable systemic infection in at least 3 cases

Discussion

Whatever would be the correct explanation for these and all available adequate clinical observations, the above trials appear to demonstrate that in different kinds of severe granulocytopenia with or without other cytopenia and with or without recovery, a great part of mature granulocytes exhibit increased alkaline phosphatase activities at the early days of successful or unsuccessful attempts to recover from granulocytopenia in the marrow as well as in the peripheral blood This means, that in these emergency situations in which, with one exception, marrow granulocyte reserves were absent, and at least at the beginning of recovery granulocyte production rate should be greatly increased along with an apparently immediate release of granulocytes from the marrow granulocytic alkaline phosphatase activities are already significantly increased

The eventual role of prednisolone causing high phosphatase scores can be excluded (cases 2 and 4) and that of systemic infection does not appear to be decisive (case 4) Chronic cases of true aplastic pancytopenia with not infrequent absence of recognized systemic infection also speak against the exclusive role of systemic infectious states Table II attempts to give a superficial information about the state of certain conditions related to changes in granulocyte alkaline phosphatase activity

There are of course, other factors which may influence the enzyme activity of the circulating granulocyte, such as their location (e g, marginal pool splenic pool) and their elimination rate and the significance of these factors must be considered in every single case

If we consider granulocyte alkaline phosphatase activity to indicate a kinetic parameter, its differential diagnostic usefulness would be less than generally supposed In fact although the majority of our 66 chronic granulocytic leukaemia cases exhibited quite low numbers of alkaline phosphatase positive cells normal or elevated counts occurred at each time period of the process (46% in its 1st year, 25% in years 2, 3, 4 5

Table II. Type of released mature marrow granulocytes related to their alkaline phosphatase activity[1]

Condition	Mature granulocyte reserves in the marrow	Type of released mature marrow granulocytes	Granulocyte alkaline phosphatase activity, direction of change towards
Normal	steady state with storage	steady state with storage	
Normal, stimulated			
e.g. endotoxin	steady state	stored	negativity
subsequent phase	with storage reduced?	newly formed	positivity
Steroid administration,			
early phase	present	stored	negativity
subsequent phase	reduced	newly formed	positivity
Surgery	present	stored	negativity
Aplastic pancytopenia	reduced or absent	newly formed	positivity
Recovery from aplastic pancytopenia or agranulocytosis	reduced or absent	newly formed	positivity
So-called hypersplenism	reduced?	newly formed	positivity
Advanced myelofibrosis	reduced	newly formed	positivity
Chronic granulocytic leukaemia	increased	stored	negativity
Acute leukaemia	reduced?	stored?	negativity
Polycythaemia vera	increased	newly formed?	positivity

[1] The uncertainty of certain statements is signed by question marks.

and 31% in years 6 and 6+) despite the exclusion of the periods of blastic transformation from the material[2].

In 5 out of 8 determinations performed of 3 chronic granulocytic leukaemia patients who were inadvertently rendered aplastic by chemotherapy, ROSNER *et al.* [7] found marked increases in enzyme level, and in at least one patient a bone marrow chromosome preparation, performed at a time when the enzyme level was markedly increased, showed the Philadelphia chromosome in all evaluable mitoses. Further, leukocyte alkaline phosphatase is able to return in chronic granulocytic leukaemia mar-

[2] These results are based on absolute counts of circulating alkaline phosphatase positive granulocytes and are therefore not directly comparable to the results which depend on scoring technique.

row cells cultured in a diffusion chamber system [8] These data appear to support my outlined concept on the meaning of the granulocyte alkaline phosphatase activity

Acknowledgment Several points of table II have been fruitfully discussed with Dr D LEHOCZKY The help of Mrs KATALIN GÁLLIK is also acknowledged with thanks

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Acta Haemat. 50: 25–29 (1973)

Spontaneous Platelet Aggregation in Myeloproliferative Disorders

A Preliminary Study

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Abstract. Spontaneous platelet aggregation has been studied both in normal subjects and in a group of patients affected by chronic myelogenous leukaemia, myelofibrosis, polycythaemia vera and thrombocythaemia. Cold-induced spontaneous aggregation occurs both in normals and in patients. Platelet-rich plasma derived from 13 out of 23 patients, spontaneously aggregated even when kept at 23 °C. Spontaneous platelet aggregation was inhibited by aspirin.

Key Words
Aspirin
Myelofibrosis
Myelogenous leukaemia
Platelet aggregation
Polycythaemia vera
Thrombocythaemia

Thrombotic and haemorrhagic complications are well documented in myeloproliferative disorders [7, 9]. Recently, Nemeth *et al.* [6] and Cardamone *et al.* [2] demonstrated some degree of platelet function abnormality in patients with bleeding diathesis. The mechanisms underlying the thrombotic complications have not been satisfactorily elucidated.

The present study seems to indicate that platelets, in some patients with myeloproliferative diseases, show an abnormal tendency to spontaneous aggregation.

Materials and Methods

Platelet-rich plasma (PRP) was prepared from venous blood collected in plastic tubes, taking care to avoid exposure to glass. The blood, anticoagulated with 1:10 of [illegible], was centrifuged at room temperature for 15 min at 600 rpm.

Platelet-poor plasma (PPP) was obtained by centrifuging PRP at [illegible] rpm for [illegible] min.

After an initial platelet count, PRP was diluted with PPP to give a platelet concentration of [illegible]/mm³.

Table I Spontaneous aggregation (SA) of PRP kept at 23°C for 2 h

	Number of cases	Platelets $\times 10^3$	White blood cells $\times 10^3$	Cases with SA	Latency time, min (mean ± SD)
Normal	15	200–300	5–8	none	
Chronic myelogenous leukaemia	10	170–600	12–152	6	7 ± 4
Myelofibrosis	5	150–300	10–15	2	12
Polycythaemia vera	7	350–500	10–18	4	7 ± 2
Thrombocythaemia	1	500	10	1	12

Each test has been carried out at least twice on different occasions.

1 ml of PRP, with known platelet number, was distributed into glass test tubes. All the glassware was silicone coated (Siliclad Clay Adams). Platelet aggregation was measured turbidometrically in Mustard's aggregometer [5] attached to a Bausch Lomb recorder. One ml sample of PRP was placed in the cuvette holder of the aggregometer which stirred the PRP at 1 000 rpm. The tube holder was kept at 37 °C. Spontaneous aggregation was tested immediately after storage for 2 h of PRP at 37, 23 (room temperature) and 4 °C (refrigerator).

When spontaneous platelet aggregation was detected in the aggregometer, this was confirmed by either gross inspection or microscopy. Platelet counting was performed according to PALUMBO and DINI [8].

15 normal subjects and 23 patients (table I) were investigated.

Results

Chilled platelets spontaneously aggregated in normal as well as in patients. Platelets of normal subjects did not aggregate when kept at room temperature or 37 °C. On the contrary, PRP derived from patients showed, in 13 out of 23, spontaneous aggregation at 23 and 37 °C (table I). Some examples of spontaneous platelet aggregation curves obtained in normals and patients are given in figure 1.

The supernatant, derived from patients' PRP kept at 23 or 37 °C, after spontaneous aggregation was still able to induce platelet aggregation of normal fresh PRP (fig. 2). Platelet aggregation of normal fresh PRP was also induced by adding the supernatant PPP derived from the normal PRP kept at 4 °C after spontaneous aggregation.

Aspirin administration in a single dose of 500 mg abolished the spontaneous platelet aggregation at 4, 23 and 37 °C in all cases.

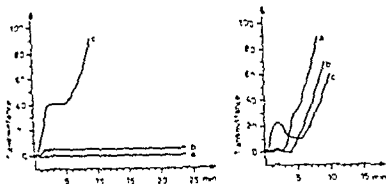


Fig. 1. Changes in transmittance during spontaneous platelet aggregation (mean of 3 determinations). On the left, normals; on the right, patients with chronic myelogenous leukaemia. a = PRP kept at 23 °C, b = PRP kept at 37 °C, c = PRP kept at 4 °C.

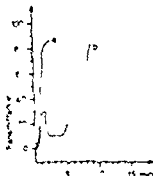


Fig. 2. Platelet aggregation curves obtained with 0.9 ml of fresh normal PRP + 0.1 ml of supernatant after aggregation (a) or 0.95 ml of fresh normal PRP + 0.05 ml of supernatant after aggregation (b).

Discussion

ZUCKER and BORRELLI [11] and ANSTALL and HAWKEY [1] reported that platelets, when incubated at low temperature, spontaneously aggregate. Recently, KATTLOVE and ALEXANDER [3] showed that cold induced aggregation resembles ADP induced aggregation in that the shapes of both aggregation curves are similar. Moreover, both aggregations are in-

hibited by the same antiaggregant compounds Cold induced aggregation was considered not to be mediated by ADP, since this compound was not found in the supernatant of chilled PRP

Our results confirm that cold induced aggregation is present both in normal and in patients affected by chronic myelogenous leukaemia myelofibrosis polycythaemia and thrombocythaemia In addition we have observed that PRP, in 56% of these patients spontaneously aggregated even when kept at 23 and 37 °C This aggregation was exactly the same as that normally seen when collagen is added to normal plasma We observed that supernatant derived from PRP at the end of the aggregation, was able to induce platelet aggregation of normal fresh PRP The kind of such curve was similar to ADP-induced curve These phenomena were inhibited by aspirin

There are no evidences to explain our findings, however, these preliminary results suggest that spontaneous platelet aggregation might underlie the thrombotic complications in patients with myeloproliferative diseases In this connection VREEKEN and VAN AKEN [10] and MUNDALL *et al* [4] demonstrated a clear relationship between abnormal spontaneous platelet aggregation and transient attacks of vascular ischaemia in 2 patients affected by polycythaemia and thrombocythaemia

Acknowledgment We are grateful to Dr F DUCKERT for his criticism in this work

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Acta Haemat 50 30-35 (1973)

Haemoglobin O Indonesia (α116 Glutamic Acid→Lysine) in an Iranian Family

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Abstract During surveys for abnormal haemoglobins in Iran a number of known and unknown variants of haemoglobin A were found In one family, a case of Hb Indonesia α116 Glu→Lys was found The presence of this haemoglobin was not associated with clinical symptoms

Key Words
Haemoglobin O
Haemoglobinopathies
Iran

Haemoglobin O Indonesia (α116 glutamic acid→lysine) was first observed by LIE-INJO and SODONO [11], in Indonesia, and it's structure was determined by BAGLIONI and LEHMANN [1] We have recently observed this haemoglobin in an Iranian family during surveys for abnormal haemoglobins on 15,000 patients from University hospitals in which a number of known and unknown variants of haemoglobin A (Hb A) were found [13, 14] It is not surprising to observe abnormal haemoglobins so frequently among Iranians, since, during centuries, many nations migrated through this country from far east and India to the near east and Europe and, after numerous invasions, groups of people remained and inhabited in some part of the country One can now trace these ancient populations by studying haemoglobin types of different ethnic groups living in Iran

Methods

The propositus is a 32 year-old man apparently healthy, no other members of his family were available for study Routine haematological examinations were within normal range according to the standard technics [18] No inclusion bodies were found in the red cells sickle cell preparation was negative Solubility test was



Fig. 1. Starch gel electrophoresis, tris-EDTA-borate buffer, pH 8.1. Above: Hb A + Hb S + Hb A_2; below: Hb A + Hb O.

within the normal range [9], and fetal haemoglobin was less than 1% [16]. Heat denaturation was negative as carried out according to GRIMES *et al.* [7] and CARRELL and KAY [3].

Results

On electrophoresis on cellulose acetate [6], the variant moves as Hb S; the proportion of the abnormal fraction, was 20% as estimated by elution of haemoglobin bands from cellulose acetate after electrophoresis [12]. In starch gel electrophoresis with tris-EDTA-borate buffer, pH 8.1, the variant moves slightly faster than Hb A_2 (fig. 1); the variant separates from Hb A after electrophoresis in agar gel at pH 6.2 [15], with the same mobility of Hb C.

The abnormal haemoglobin was purified by column chromatography of the haemolysates on DEAE-cellulose (Whatman DE-52), using tris-HCl buffer, pH 8 and 0.05 M [8]; the variant was separated from Hb A_2 and obtained in a pure form. Globin was prepared by the treatment of the purified Hb O with 2% concentrated HCl in acetone at –20 °C, washed with cold acetone and freeze-dried.

To identify the abnormal polypeptide chain, electrophoresis of the globin was carried out in starch gel containing 6 M urea and 0.05 M 2-mercaptoethanol [4], which revealed an additional slow moving α-chain

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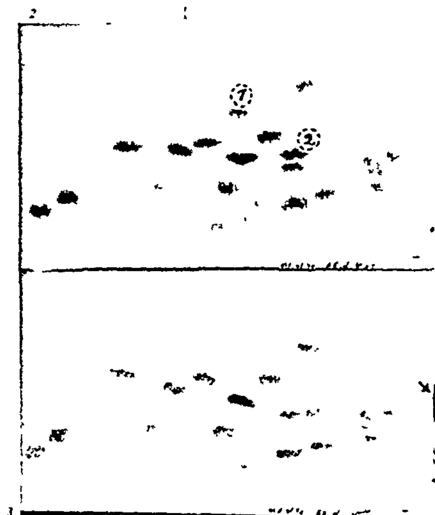


Fig 2 Electrophoresis of the globin in starch gel containing 6 M urea and 2 mercaptoethanol Note the additional slow moving α-chain

Fig 3 Fingerprints of the S aminoethylated α chain from Hb O (above) and Hb A (below) Arrow indicates the usual place of the peptide αTp 12b 1 and 2 are 2 new peptides which both stain for histidine

Table I

Amino acid	Peptide 1	Peptide 2
Lysine	1	2
Histidine	1	1
Aspartic acid	–	1
Threonine	1	1
Serine	–	1
Proline	1	1
Alanine	3	2
Valine	1	1
Leucine	4	1
Phenylalanine		1

(fig 2) suggesting that the abnormality rested in the α-chain. This was confirmed by the electrophoresis of the haemolysates on cellulose acetate [17] as well as treatment of the haemolysates with p-hydroxymercuribenzoate (PMB) [2], followed by starch gel electrophoresis, pH 8.6.

α Polypeptide chain from Hb O was prepared by CMC column chromatography with phosphate buffer, pH 6.7, containing 6 M urea and 0.05 M 2-mercaptoethanol followed by aminoethylation [5]. Tryptic digestion of S-aminoethylated normal and abnormal α-chain and fingerprinting was carried out according to CLEGG *et al.* [5], except that isoamyl pyridine H_2O (30:30:35) was used for chromatography. Figure 3 shows the fingerprints of the aminoethylated peptides from Hb O as well as normal α-chain. The peptide αTp 12b is missing from it's usual place (arrow) and 2 new peptides, 1 and 2, are present in the fingerprint of Hb O, both of these peptides stain for histidine.

The abnormal peptides were each eluted from 6 preparative fingerprints with 6 N HCl and hydrolysed for 20 h at 105 °C in microcaps. HCl was removed by rotary evaporation and their amino acids were determined on an automatic amino acid analyser (Hitachi KLA-5). Table I shows the amino acid contents of the 2 extra peptides 1 and 2.

The results of the amino acid analysis of the 2 extrapeptides indicate that in this variant residue 116 of the α-chain glutamic acid has been replaced by lysine and after tryptic digestion this lysine has been cleaved by trypsin resulting the peptide αTp 12b to split to 2 new peptides which are present in the fingerprint of Hb O: 1 and 2 in figure 3.

The amino acid composition of the extra peptide 1 is the same with the first part of the peptide αTp 12b, namely residue 104–116, except glutamic acid which is replaced by a lysine, and the amino acid composition of the extrapeptide 2 is similar to residue 117–127 of the α-chain

The substitution of a lysine for a glutamic acid has rendered Hb O to have 2 additional positive charges from Hb A, as detected from it's electrophoretic mobility at alkaline pH

Acknowledgement This work was supported partly by Research Grant No 313 50 from the Ministry of Science and Higher Education of Iran

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Acta Haemat. 50: 36–43 (1973)

Übergang einer Polycythaemia vera in eine akute Monozytenleukämie

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Abstract. In a 68 year old male patient a polycythemia vera underwent a transformation into an acute leukemia. The dominant cells could be identified as monocytes by cytochemical staining. This observation can be used as sign of the myelogenic origin of the monocytes.

Key Words
Cytochemistry
Leukemia following polycythemia
Monocyte origin
Monocytic leukemia
³²P therapy
Polycythemia

Übergänge der Polycythaemia vera (PV) in eine akute Leukose werden nach Radio-Phosphor-Therapie in 10–14% der Fälle beobachtet [7, 21, 22]. Es ist umstritten, ob es sich hierbei um einen direkten Effekt der ionisierenden Strahlen handelt [12, 13], oder ob durch die bessere Lebenserwartung die Chance wächst, das der Krankheit innewohnende Risiko der Transformation in die Wucherung unreifer Zellen zu erleben [14, 15].

Die Leukosen werden in der Regel als «akute myeloische Leukämien» [2, 9], zum Teil auch als «Paramyeloblastenleukämien» [16, 19] bezeichnet. Zu unserer Überraschung konnten wir in einem Fall die sich aus einer PV entwickelnde akute Leukose als Monozytenleukämie klassifizieren. Zytochemische Untersuchungen über entsprechende Krankheitsverläufe sind uns nicht bekannt. Lediglich wurde im Einzelfall bei einer chronischen Lymphadenose der Übergang in eine Monozytenleukämie beschrieben [4]. So möchten wir über diesen Krankheitsverlauf berichten, zumal er einen weiteren Beitrag zur Diskussion um die Herkunft der Monozyten darstellt.

Krankengeschichte

G. B., geb. 1905, Krbl. 12543. Familienanamnese unauffällig. 1929 Appendektomie, 1941 Tonsillektomie, Cholezystitis. 1956 wurde der Verdacht auf eine Polycythaemie geäussert. Im November 1957 erstmals Untersuchung in der Medizinischen Universitäts-Klinik Göttingen. Etwas reduzierter Allgemeinzustand. Ausgeprägte Rubrozyanose, vermehrte Blutfülle der Schleimhäute. Leichte Vergrösserung der Leber (+2 QF). RR 200/120 mm Hg. Hb 22,5 g%, Erythrozyten 7,92 Millionen/mm³, Hämatokrit 80%, Thrombozyten 620 000/mm³, Leukozyten 10 900/mm³ (Stab. 8, Seg. 58, Eo 3, Baso 0, Lympho 24, Mono 7%).

Im September 1966 Bestätigung der Diagnose durch histologische Untersuchung einer Beckenkammbiopsie (Abb. 1) sowie durch Bestimmung des Erythrozyten- und Plasmavolumens. Vom November 1957 bis März 1972 Behandlung mit wiederholten Aderlässen und insgesamt 40 mCi ^{32}P, verteilt auf 9 Einzeldosen. Nach der Behandlung bis 1971 immer wieder vorübergehende Besserung der durch die PV verursachten Beschwerden. Seit 1967 rezidivierende Thrombophlebitiden. 1969 Lungenembolie. 1969 Nachweis einer sekundären Hyperurikämie mit Uratsteinen. Seit 1970 leichte Retention harnpflichtiger Substanzen. 1970 Diagnose einer Au-Ag-positiven Hepatitis durch Leberblindpunktion.

Im März 1972 erneute Aufnahme wegen Atemnot, Kopfschmerzen, Nachlassen der Leistungsfähigkeit. Klinisch trotz Digitalisierung Zeichen der Herzinsuffizienz, ausserdem nach wie vor Aspekt eines Vollbildes der PV: Rubrozyanose, injizierte Konjunktiven, Milztumor (+2 QF). Hb 20,1 g%, Erythrozyten 9,64 Millionen/mm³, Hämatokrit 70%, Thrombozyten 365 000/mm³, Leukozyten 10 400/mm³ mit Linksverschiebung (10% Stab.).

In der Beckenkammbiopsie war die Granulozytopoese gegenüber 1966 in der Reifungsreihe stärker linksverschoben. Im übrigen wie früher enorm gesteigerte Zelldichte durch Vermehrung vorwiegend der erythropoetischen Zellen, aber auch der zum Teil in Nestern liegenden Megakaryozyten (Abb. 2). Erneute ^{32}P-Therapie (4 mCi). Bei gleichbleibendem Befinden im Mai 1972 Abfall des Hb auf 14,4 g%, der Leukozyten auf 2400/mm³, im Differentialausstrich erstmals 16% unreife Zellen.

Juli 1972 Hohes Fieber, allgemeine Schwäche. Zunehmende Hepatosplenomegalie. Hb-Abfall auf 9, später 7 g%. Anstieg der Leukozyten auf 20 000–50 000/mm³ (5) bis zu terminal 98% Blasten. Im Knochenmark nahezu ausschliesslich grosse unreife Zellen, deren Kern ein lockeres Chromatingerüst und mehrere grosse Nukleolen enthält und deren Zytoplasma sich nur ungleichmässig anfärbt. Bei der zytochemischen Analyse enthalten diese Zellen reichlich [illegible] esterasepositive Granula, dabei nur gelegentlich eine schwach positive Reaktion auf Peroxidase. PAS-Reaktion negativ (Abb. 3). (Naphthol-AS-Esterase-Färbung nach Löffler [10], Peroxydase nach Graham-Knoll und PAS-Reaktion [3].) Trotz kombinierter zytostatischer Behandlung Verschlechterung des Allgemeinzustandes mit terminalem Blastenanstieg auf Werte um 300 000/mm³. Am 24. September 1972 Exitus im Kreislaufverfall. Die eine Stunde post mortem durchgeführte Beckenkammbiopsie zeigte eine diffuse Durchsetzung des Knochenmarks mit unreifen rundkernigen Zellen, deren Kern zum Teil leicht gebuchtet erscheint, normale blutbildende Zellen fehlen (Abb. 4).

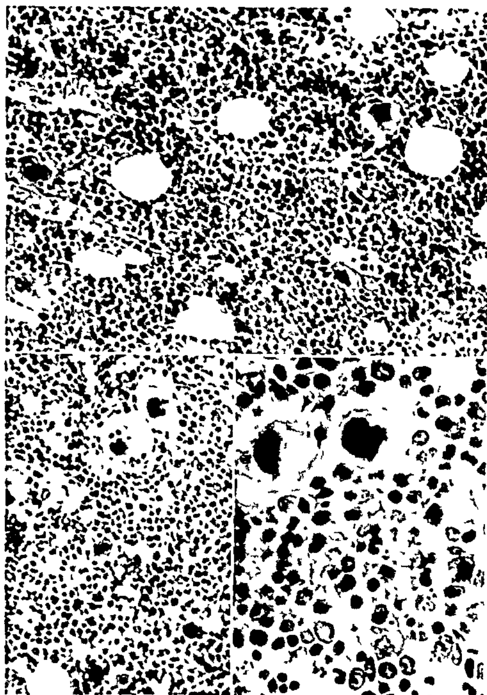


Abb. 1. Beckenkammbiopsie 1966. Gesteigerte Zelldichte durch Hyperplasie der Erythro-, Granulo- und Thrombopoese. Weite Sinusoide. HE, × 270

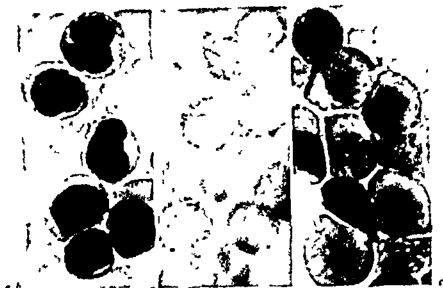


Abb. 1. Blutausstrich im terminalen Blastenschub. *a* Pappenheim-Färbung. In den zum Teil gebuchteten Kernen grosse Nukleolen. *b* Positive Esterasereaktion. *c* Peroxydasereaktion nur vereinzelt schwach positiv. Zum Vergleich stark positive Reaktion bei einem Segmentkernigen (oben). × 1000

Sektionsbefund Nr. 601/72: Rote Metaplasie des Femurschaftmarkes. Hepatosplenomegalie (Leber 2?00 g, Milz 1900 g). Lymphknotenschwellungen paraaortal bis hühnereigross. Zentral ulcerierte [illegible] Tumorknoten in Sigma und Rektum. Mikroskopisch: In Femurschaftmark, Milz, Lymphknoten eine dichte Durchsetzung mit unreifen Zellen wie in Abbildung 4. Gleichartige Zellinfiltrate tumorförmig in Sigma und Rektum, periportal betont in der Leber, ferner herdförmig in den Nebennieren und den Nieren.

Nebenbefunde: Exzentrische Linksherzhypertrophie. Nephrolithiasis. Chronische Pyelonephritis mit Basalmembranverdickungen der Glomerulumschlingen und periglomerulärer Fibrose. Chronische Cholezystitis. Periportale Fibrose in der Leber mit Gallengangswucherungen und lockeren Rundzellinfiltraten.

Abb. 2. Beckenkammbiopsie März 1972: Weitgehend erhaltene [illegible] Zelldichte durch Hyperplasie vorwiegend der Erythro- und Thrombozytopoese mit [illegible] Granulopoese(?). [illegible] Zellen treten gegenüber den erythropoetischen Zellen [illegible] und sind innerhalb der [illegible] stärker [illegible]. [illegible] gegenüber Abb. 1 [illegible] starke Zell[illegible] bei [illegible] überwiegt [illegible]. [illegible] × 480

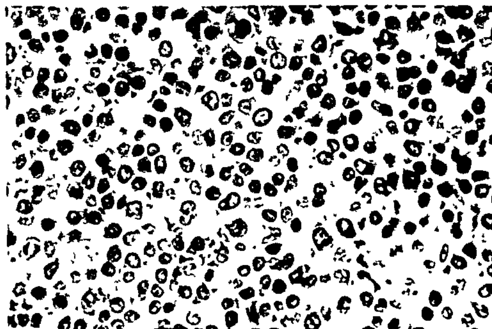


Abb. 4. Unmittelbar postmortal entnommene Beckenkammbiopsie. Dichte Durchsetzung des Knochenmarks mit unreifen Zellen. Goldner × 490

Diskussion

Nach 16jährigem Verlauf ging bei diesem Patienten die PV in eine akute Leukämie über. Bei der zytochemischen Differenzierung der Blasten sprach der Nachweis der unspezifischen Esterase unter Verwendung von Naphthol-AS-Azetat als Substrat bei nur sehr schwach positiver Peroxydase- und negativer PAS-Reaktion dafür, dass es sich um eine Monozytenleukose handelte [1–3, 11, 18]. Bei der Pappenheim-Färbung wie auch bei der histologischen Untersuchung fielen nur gelegentlich leichte Kerneinbuchtungen auf. Im übrigen entsprach der postmortale Befund dem anderer terminaler Leukosen bei PV. Weitere zytochemische Untersuchungen, Hautfensteruntersuchungen oder die Lysozymbestimmung im Serum und Harn [17, 18] waren wegen des raschen Verlaufes nicht mehr möglich.

Im Schrifttum ist man kaum auf die Frage eingegangen, um welche Art von Blasten es sich im Terminalstadium einer PV handelt. Es wird von Paramyeloblasten [16], auch von monozytoiden Blasten [14, 19] ge-

sprochen, ohne dass dieser Eindruck durch zytochemische Untersuchungen belegt werden konnte. Bei unserer Beobachtung handelte es sich um Zellen, die zytochemisch als Monozyten zu differenzieren waren. Man muss also annehmen, dass nach der zur Ausreifung befähigten Proliferation *aller* blutbildenden Zellen eine zweite Krankheitsphase folgte, in der allein Monozyten gebildet wurden bzw. die zur Proliferation stimulierten Stammzellen sich allein zu Monozyten entwickelten.

Bei der Diskussion, ob die Monozyten den myeloischen Zellen im engeren Sinne zuzurechnen sind [Literatur s. 8, 17] oder ob sie dem retikuloendothelialen System (RES) entstammen [Literatur s. 16], ist diese Beobachtung als ein weiterer Hinweis auf die myelogene Entstehung der Monozyten zu werten. Es ist naheliegend, dass die zuvor gesteigerte Proliferation myeloischer Zellen auch terminal im undifferenzierten Wachstum myeloischer Zellen endet. Das gilt besonders, da bei der PV das gleichzeitige oder terminale Vorkommen vom RES ausgehender Tumoren oder Systemerkrankungen nicht beschrieben wurde [2, 5, 19, 20].

Die Phase der Umwandlung der PV in eine akute Leukose wird nur ausnahmsweise bei der Knochenmarkuntersuchung erfasst. Auch in diesem Fall wurde das Mark nur vor oder nach der Krankheitstransformation untersucht. 5 Monate vor dem Tode, als die Erythropoese noch maximal stimuliert war, fiel im Knochenmark eine stärkere Linksverschiebung der granulopoetischen Reifungsreihe auf, ohne dass im Blutausstrich unreife Zellen nachweisbar waren (vgl. Abb. 2). So ist es möglich, dass die Blastenwucherung im hyperplastischen Mark begann. Da jedoch der massiven Blastenausschwemmung ein [illegible] der Zytopenie vorausging, ist als zweite Möglichkeit zu diskutieren, dass die Proliferation der Blasten in einem nach der letzten Radiophosphortherapie phthisisch umgewandelten Mark begann, wie wir es früher zufällig bei einer histologischen Untersuchung erfassen konnten [6]. Schon wegen des engen zeitlichen Zusammenhanges ist es in diesem Fall wahrscheinlich, dass die Krankheitsumwandlung durch die unmittelbar vorausgegangene Radiophosphortherapie begünstigt wurde.

Zusammenfassung

Bei einem [illegible]jährigen Patienten entwickelte sich aus einer Polycythaemia vera nach [illegible]jährigem Verlauf eine akute Leukose. Die Blasten konnten zytochemisch als Monozyten differenziert werden. Diese Beobachtung ist als weiterer Hinweis auf die Entstehung der Monozyten aus myeloischen Zellen anzusehen.

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Acta Haemat. 50: 44–49 (1973)

Ropalocytosis in a Patient with Acute Lymphoblastic Leukemia

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Abstract. A patient with acute lymphoblastic leukemia whose red blood cells showed the abnormality ropalocytosis is described. This abnormality is characterized by bizzare pseudopodia-like processes detectable in the mature red blood cells and reticulocytes. The presence of ropalocytosis suggests pathology of the hematopoietic system.

Key Words
Erythrocyte morphology
Leukemia
Lymphoblastic leukemia
Ropalocytosis

Alterations of the red blood cell morphology in leukemia, such as anisocytosis, macro- and microcytosis, poikylo- and spherocytosis are well known. Recently, an unusual abnormality of the red blood cells in hairy cell leukemia was reported by GHADIALLY and SKINNIDER [5] and named by the authors ropalocytosis [4]. The authors define this abnormality as consisting of numerous branched and unbranched cell processes protruding from the cell membrane. They can be observed in ultrathin sections examined with the electron microscope.

We had the opportunity to examine a patient with acute lymphoblastic leukemia whose red blood cells showed alterations compatible with these described as ropalocytosis.

Case Report

A 67-year-old German-born male was admitted to the department on September 7, 1972, because of a painful ulcer in the inner part of the upper lip. The lesion appeared 1 month prior to admission, healed after a few days without any treatment and reappeared after 2 weeks.

Past history revealed a partial gastrectomy performed 20 years previously because of duodenal ulcer and mild diabetes mellitus kept under control by diet only.

On physical examination, the patient was found to be in poor general condition. Blood pressure was 150/70 mm Hg. On the left side of the upper lip an ulcer could be seen 3 cm in diameter, having regular margins, a white bottom and no hemorrhages. Except for a few palpable lymph nodes in the left submandibular region, 1 cm in diameter, not tender and soft, there were no additional pathological findings.

Laboratory examinations showed hemoglobin 8.1 g%, HTK 25%, reticulocytes 2.2%, total white blood cell count 2,200/mm³, with the following differential count: polymorphonuclears 4%, basophils 2%, monocytes 1%, lymphocytes 80% and blasts 13%. Platelet count 35,000/mm³. The blood smear revealed marked anisocytosis, macrocytosis and a few spherocytes.

Bone marrow aspiration biopsy revealed a slightly hypoplastic bone marrow, multinuclearity of the cells of the red blood series, marked hypoplasia of the granulocytic series and infiltration by the same lymphocytes and blasts seen in the peripheral blood smear. Serum iron was 163 µg%, serum iron binding capacity 120 µg%, bilirubin 1.0 mg%, unconjugated 0.7 mg%. Direct and indirect Coombs' tests were negative; the plasma hemoglobin concentration was 2.5 g%. Urea, glucose, creatinin, uric acid, cholesterol, total proteins, albumin, globulin, protein pattern by paper and immunoelectrophoresis, fibrinogen, prothrombin, SGOT and SGPT, were normal. The 24 hour stercobilinogen excretion was 132 mg%.

The diagnosis of acute lymphoblastic leukemia was considered. Treatment with prednisone (40 mg/day) and vincristine (2 mg/m²) was started. Two days following the biopsy, the patient started to bleed profusely from the operative wound, as well as from the nose. A survey of the coagulation mechanism revealed a reduced euglobulin lysis time of 60 min (normal value 120 min), the presence of fibrinogen degradation products (40 µg/ml), PTT 37 sec (normal 35–45 sec) and fibrinogen 280 mg% (normal 400–600 mg%). Treatment with 16 g/day of amicar (epsilon aminocaproic acid) in addition to repeated blood transfusions stopped the bleeding. The patient's condition improved, but a few days later his temperature rose and moniliasis appeared in the buccal mucosa; administration of amphotericin B and mycostatin did not result any improvement and death occurred 27 days after the admission. Post mortem examination was denied by the family.

Electron microscopy. Buffy coat was obtained from 10 ml venous blood withdrawn in a heparinized syringe, fixed in 1% cold glutaraldehyde, pH 7.4, for 2 h and postfixed in 1% osmium tetroxide. The cells were dehydrated in graded alcohols and embedded in epon 812. Thin (500–600 Å) sections were cut with an LKB ultratome III and examined with a Philips 201 electron microscope.

Some of the red blood cells and reticulocytes revealed a bizarre and complicated shape (fig 1). They showed pseudopod-like processes with clubbed ends, mostly protruding from a limited portion of the cell membrane, although in a few instances complete irregularity of the cell membrane was noted (fig 2). In some of the cells (fig 3) concentric rings were demonstrated, whereas other cells showed numerous and large vacuoles, a few containing cytoplasmic debris. They were ob-

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Acta Haemat 50 50 55 (1973)

The Effects of Irradiation on the Haematopoietic Tissues of Anaesthetized Mice

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Abstract The effects of irradiation on the haematopoietic tissues of anaesthetized mice have been measured using the balance between damage and recovery during the 10-day period following the administration of various doses of whole body X irradiation The cellularity of the femoral bone marrow the weight of the spleen and the number of (endogenous) spleen colonies are elevated in animals anaesthetized using Avertin during exposure to doses of whole body X irradiation in the range between 400 and 850 rad with a dose reduction factor of 1 2 1 4

Key Words
Anaesthesia
Bone marrow
Irradiation
Lymphoid tissues
Mouse haematopoiesis
Spleen colonies

Studies of the radiosensitivity of haematopoietic stem cells and of haematopoietic recovery in partially irradiated animals which can provide extremely useful information, often necessitate the irradiation of immobilized, anaesthetized animals [2, 3, 6] It is, therefore, important to recognise the possibility that anaesthetics which have not been subjected to systematic evaluation may distort the effects of irradiation [1] A weak protective effect has been observed after the administration of ethyl alcohol but this could not be attributed to its anaesthetic properties and neither nembutal nor ethyl carbamate modified the survival patterns of irradiated mice [7]

VAN BEKKUM [13] has however, reported an increase in the LD 100 30d minimum in mice which were immobilized while they were exposed to whole body X-irradiation Subsequent investigations have revealed that both physical restraint and Nembutal anaesthesia enhance the survival of bone marrow CFU, while several other anaesthetics increase the LD 50 30d without influencing the gastro intestinal syndrome [4, 5]

The effects of irradiation on the haematopoietic tissues of mice anaesthetized using Avertin have been studied in the present investigation, which forms part of a series of experiments designed to analyse the effects of whole body X-irradiation on the haematopoietic and lymphoid tissues of the mouse.

Materials and Methods

Animals and housing. Specific pathogen free female albino mice of the CSI strain (supplied by Scientific Products Farm, Ash, Kent) weighing between 20 and 25 g were used throughout this investigation. The mice were housed 5 to a cage in a laminar air flow cabinet and given boiled water and mouse diet 41B ad libitum.

Irradiation and anaesthetic. Mice were irradiated in the radially disposed compartments of a circular cage (300 kVp, 5 mA, 0.5 mm copper plus 5 mm aluminium filtration, dose rate 64 rad/min).

Avertin with amylene hydrate (tribromoethanol, Winthrop Laboratories, New York) was made up in warmed isotonic saline and 0.01 ml/g body weight (300 mg of Avertin/kg body weight) was injected intraperitoneally to anaesthetise the mice for the duration of the irradiation.

Experimental design. Eight groups of not less than 10 mice received doses of X-irradiation in the range between 200 and 850 rad. The LD 50/30 for CSI mice is 700 rad. Five groups of not less than 7 anaesthetized mice were also irradiated. After 19 days, the irradiated mice were killed by exposure to ether vapour. The spleen, thymus, inguinal lymph nodes and brachial lymph nodes were dissected and weighed. The cellularity of the femoral bone marrow was measured by syringing the contents of the medullary cavity of the femoral diaphysis into 20 ml of Isoton and determining the cell content of the resultant suspension using a Coulter Counter. After the spleen had been fixed in aqueous Bouins fluid it was transferred to 70% alcohol and macroscopic surface colonies were subsequently counted (9

Results

The average numbers of nucleated cells in the femoral diaphysis and the weights of the spleen, thymus and pooled lymph nodes have been plotted for the control and anaesthetised groups of irradiated mice (Fig. 1–4). In both groups all of these parameters decrease as the dose of X-irradiation is increased. The cellularities of the femoral bone marrow and the weight of the spleen are, however, elevated in the groups which were irradiated under Avertin anaesthesia ($p<0.05$ for both at 670 rad). The dose reduction factor (DRF) in the range between 400 and 850 rad is about 1.2–1.4 for these tissues. The number of endogenous spleen

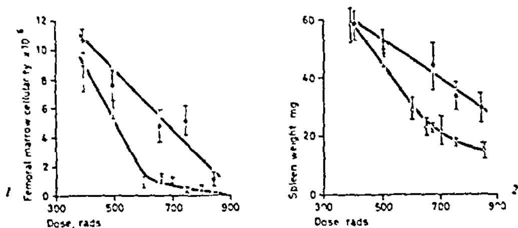


Fig 1 The cellularity of the bone marrow 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole body X irradiation

Fig 2 The weight of the spleen 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole body X irradiation

colonies is similarly elevated with a similar DRF (fig 5) Most of the spleens in the anaesthetized mice which received 500 rad of whole-body X-irradiation exhibited confluent areas in which individual colonies could not be discerned, whereas in the control group the surface colonies were not confluent after this dose of X-irradiation The weight of the thymus was elevated relative to the weight of the thymus in controls only in anaesthetized animals exposed to the highest doses of X-irradiation administered, and the weight of the lymph nodes was not affected by Avertin anaesthesia ($p > 0.45$ and 0 9 at 670 rad)

Discussion

Avertin anaesthesia modified the effects of whole-body X-irradiation on the haematopoietic and lymphoid tissues of the mouse in a manner which is comparable to that recently reported for several other anaesthetics [4, 5] The CFU content of the femoral bone marrow is elevated in animals anaesthetized with Nembutal before being irradiated and it has been suggested that the beneficial effect of the anaesthetic may be due to hypoxia [4, 5] In the present investigation Avertin anaesthesia has been

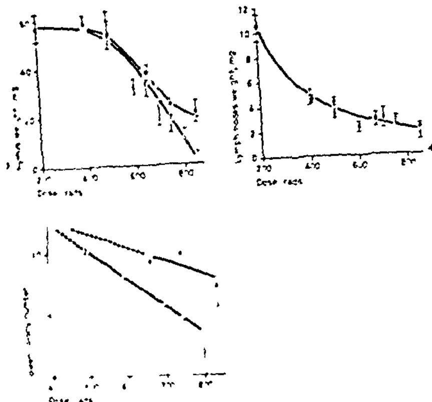


Fig. 3. The weight of the thymus 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole body X-irradiation.

Fig. 4. The weight of the lymph nodes 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole body X-irradiation.

Fig. 5. The number of macroscopic colonies observed on the surface of the spleen 10 days after the exposure of mice anaesthetized using Avertin (●) and of unanaesthetized controls (○) to various doses of whole-body X-irradiation.

shown to exert beneficial effects on the cellularity of the bone marrow and the mass of the spleen as well as on the number of colonies formed in the spleen. The thymus, which has been shown to retain its capacity for regeneration after the administration of whole-body doses of X-irradiation that inhibit regeneration in the bone marrow, the spleen and the

lymph nodes [10–12], benefits only slightly from Avertin anaesthesia during the period of irradiation. The mass of the lymph nodes is not elevated appreciably in anaesthetized irradiated animals in contrast to the cellularity of the bone marrow and the mass of the spleen. This contrast may reflect the absence of regeneration in the lymph nodes after the administration of all doses above 415±40 rad during the 10 day period following whole body X irradiation [8], so that regeneration would not be expected to occur in the lymph nodes unless the dose of X-irradiation administered was rendered comparable in its effects to a dose of less than 400 rad.

It is thus essential to take into account the actions of anaesthetics in order to be able to utilize the effects of irradiation on the haemotopoietic tissues in assay systems which depend upon the use of anaesthetized irradiated mice. It would be useful to compare the efects of Avertin and of similar anaesthetics on the haematopoietic tissues with their effects on the immune response.

Acknowledgements. This investigation was supported by a grant from the Medical Research Council of Geat Britain. We are indebted to MISS MARGARET HOWDLE who prepared the illustrations and to MISS ANN HARRIS who prepared the manuscript.

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Acta Haemat. 50: 56–63 (1973)

Morphologie de l'érythropoïèse chez *Lacerta muralis* (Laurenti)

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Abstract. Although the erythrocytes of lizard are clearly different from those of mammals (persistence of the nucleus and of a residue of cytoplasmic basophilic substance), there is an analogy in their production starting with a large pro-erythroblast. 4 successive mitoses reduce the nuclear size to $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ and $\frac{1}{16}$, allowing differentiation of 5 cell types. The passage from the marrow into the blood, however, is less selective than in mammals: in the blood of lizard one finds numerous cells that have not undergone all the mitoses and transform directly into larger erythrocytes with larger nuclei, than the majority of erythrocytes.

Key Words: Erythroblaste · Karyokinetics · Lizard erythropoiesis

Les différents stades de l'érythropoïèse sont classiquement définis chez les mammifères au moyen de critères morphologiques et tinctoriaux. Quatre stades sont décrits: proérythroblaste à gros noyau nucléolé et à cytoplasme basophile, érythroblaste polychromatophile et érythroblaste acidophile. Chez les autres vertébrés, les stades sont superposables ainsi qu'il ressort des études de YOKOYAMA [15], d'ANDREW, d'IORIO [6] chez les poissons, de DE VILLIERS-PIENAAR chez les reptiles, et de LUCAS et JAMROZ [10] chez les oiseaux.

D'intéressantes précisions ont été apportées chez l'homme par l'étude des dimensions nucléaires ou caryométrie. LEIBETSEDER [9], WEICKER [13] puis CAZAL [1], appliquant à l'érythropoïèse la méthode générale proposée par JAKOBI [7], constatent que la maturation se fait en 5 stades et non en 4, séparés par des mitoses qui divisent chaque fois par deux le volume nucléaire sans que celui-ci se reconstitue (mitoses hétéroplastiques). Les volumes nucléaires de ces 5 stades sont dans les proportions 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ et $\frac{1}{16}$. La cellule du premier stade paraît subir une

mitose hémoplastique, une des cellules filles accroissant son noyau pour reconstituer la cellule-mère, l'autre cellule-fille conservant un volume nucléaire réduit de moitié. Le cytoplasme des stades caryométriques 2 et 3 est basophile, celui du stade 4 est polychromatophile et celui du stade 5 est acidophile. Si l'on conserve donc la terminologie classique, il faut distinguer deux érythroblastes basophiles et non plus un seul.

La présente communication résume les constatations analogues faites chez le lézard et précise certaines particularités.

Matériel et Méthodes

L'étude a porté sur de jeunes lézards des murailles, *Lacerta muralis* (Laurenti) mesurant 5 cm de la tête au cloaque; les individus de taille supérieure sont éliminés car le canal diaphysaire de leurs os longs contient une trop forte proportion de moelle jaune.

Le sang est prélevé à la queue de l'animal, soit directement sur la lame, soit à l'aide d'une pipette capillaire dont la paroi interne est recouverte d'une couche d'héparine desséchée.

Pour obtenir la moelle, la diaphyse des os longs est séparée des épiphyses et, à l'aide d'une seringue adaptée à une extrémité, la moelle diaphysaire est refoulée sous pression sur une lame.

Les étalements de sang et de moelle, aussi réguliers que possible, sont colorés par la méthode panoptique de May-Grünwald-Giemsa. La substance granulofilamenteuse basophile est mise en évidence par la coloration vitale au bleu de crésyl brillant.

Les mensurations cellulaires sont réalisées sur microphotographies à grossissement [illegible]. Les surfaces sont mesurées au planimètre. Les rapports nucléoplasmiques correspondent à des rapports de surface

$$\frac{\text{surface nucléaire}}{\text{surface totale de la cellule}}$$

La caryométrie peut être réalisée à partir de mesures linéaires ou planimétriques. Ces deux procédés sont [illegible] un grand nombre de cellules érythroblastiques pris au hasard sur les microphotographies sont mesurées et réparties en classes [illegible].

Si la forme du noyau n'est pas tout à fait circulaire, le diamètre retenu est la moyenne [illegible] de ses diamètres orthogonaux. Les noyaux ovales, dont les diamètres orthogonaux diffèrent entre eux de plus de 18 %, sont cependant [illegible] comme le [illegible] [illegible]. [illegible] de cette [illegible] sur le [illegible], les courbes de [illegible] sont [illegible] que celles obtenues par la [illegible] des surfaces.

Les [illegible] sont [illegible] d'une échelle [illegible] à partir d'un [illegible] de [illegible].

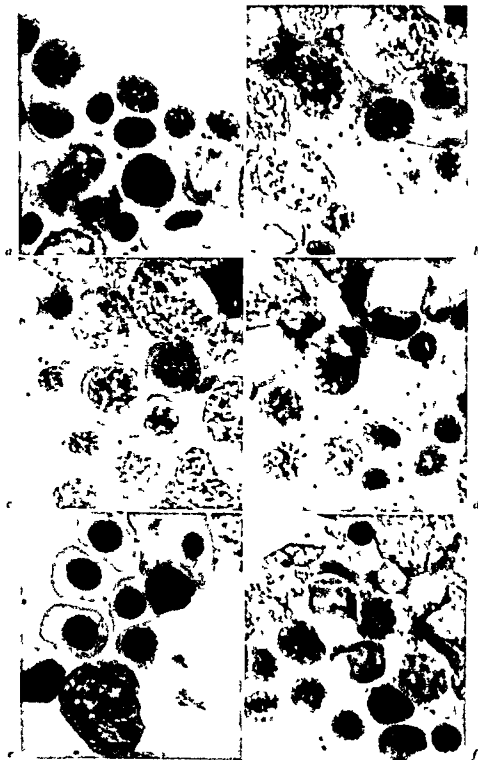


Fig. 1

Résultats

La lignée érythrocytaire dans la moelle

Les frottis de moelle contiennent des érythroblastes et des érythrocytes (fig 1). La distinction entre eux est facile en raison du noyau allongé et de la grande taille cytoplasmique des érythrocytes. Pour les courbes caryométriques, seuls les érythroblastes ont été pris en considération.

Les deux courbes de fréquence obtenues par l'étude statistique de 200 cellules peuvent être interprétées de façon identique et permettent de différencier les stades évolutifs qui se marquent par des pics de la courbe mieux délimités sur la courbe des surfaces (fig 2). Cinq pics sont identifiables, correspondant à 5 stades érythroblastiques, tout comme chez l'homme. Un simple calcul montre que le volume nucléaire de chacune de ces classes correspond à la moitié de celui de la classe suivante.

Ces 5 stades seront désignés ici par les noms de proérythroblaste, érythroblastes I, II, III, et proérythrocyte. Leur identification métrique permet d'en décrire les caractères morphologiques (fig 1). Des mitoses sont repérables dans les 4 premiers stades, mais non dans le 5e, d'où son nom impliquant une transformation sans mitose en érythrocyte.

Le proérythroblaste est le plus volumineux (diamètre 12–14 μm), le noyau circulaire ou ovalaire, central, a un diamètre moyen de 10,7 μm, son réseau chromatinien est fin et régulier, et il contient 2–5 nucléoles de limite peu nette, le cytoplasme homogène, franchement basophile avec un liséré périnucléaire plus clair, est peu abondant (rapport N/P de 0,8 environ).

Fig. 1. La lignée érythroblastique (frottis médullaire, MGG, × 630). *a*: Un proérythroblaste au centre, un grand érythroblaste basophile en haut et à gauche, un érythroblaste polychromatophile en haut et à droite, un érythrocyte en bas et à droite. *b*: En bas et de gauche à droite: une mitose proérythroblastique, 2 grands érythroblastes basophiles. *c*: Au centre: 2 grands érythroblastes basophiles et un érythroblaste polychromatophile. *d*: 3 petits érythroblastes basophiles en bas et à gauche, 2 érythroblastes polychromatophiles au centre droit. *e*: 4 érythroblastes polychromatophiles. Un érythroblaste acidophile ou proérythrocyte en voie de [illegible] noyau légèrement ovalaire, cytoplasme en cours de développement. *f*: Un érythroblaste basophile en haut, une mitose d'érythroblaste polychromatophile au [illegible] 2 érythroblastes polychromatophiles en bas au centre et à droite.

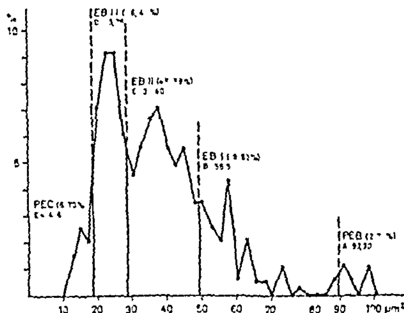


Fig 2 Fréquences érythroblastiques médullaires selon les surfaces nucléaires Les 5 stades érythroblastiques sont caractérisés par leur surface moyenne respective A B C D E PEB = Proérythroblaste, EB I = grand érythroblaste basophile EB II = petit érythroblaste basophile, EB III = érythroblaste polychromatophile PEC = érythroblaste acidophile ou proérythrocyte

L'érythroblaste I est plus petit, le diamètre nucléaire moyen est de 8,5 µm, les nucléoles ont disparu et le réseau chromatinien a tendance à se condenser en certains points, le cytoplasme est franchement basophile le rapport N/P est de 0,7

L'érythroblaste II a un noyau plus réduit (diamètre moyen 6,8 µm) à chromatine condensée en petites mottes bien distinctes, le cytoplasme est basophile le rapport N/P est de 0,55

L'érythroblaste III a un cytoplasme polychromatophile, ce qui signe la diminution de la substance basophile cytoplasmique (ARN) et l'apparition de l'hémoglobine, le noyau a un diamètre moyen de 5,3 µm, sa chromatine est condensée en mottes régulières, le rapport N/P est de 0,5

Le proérythrocyte est la cellule la plus petite de la lignée, plus petite même que les érythrocytes dont la condensation nucléaire est largement compensée par l'accroissement cytoplasmique, son diamètre varie entre 6 et 8 µm son diamètre nucléaire moyen est de 4,5 µm, la chromatine est condensée en quelques gros blocs réguliers, le cytoplasme est acidophile le rapport N/P dépasse encore 0,4

La fréquence médullaire des 5 stades n'est pas superposable à ce que l'on constate chez l'homme où le nombre double lorsque le volume nucléaire est divisé par deux. Si le même rapport est retrouvé pour les érythroblastes I et II (20 et 40 %), l'érythroblaste III est moins abondant que l'érythroblaste II (26 % seulement) et le proérythrocyte est rare (6,7 %). Ces chiffres indiquent soit qu'une partie de la production médullaire fuit vers le sang, soit que la durée des différents stades diminue avec leur degré de maturation, les derniers étant les plus courts.

La lignée érythrocytaire dans le sang

L'érythrocyte est une grande cellule elliptique, de 13 à 15 µm de long sur 7,5-9 µm de large. Le cytoplasme est très abondant (rapport N/P 0,16). Le noyau est allongé dans le même sens que la cellule, sa longueur est de 5,9 ± 0,2 µm, sa largeur de 2,54 ± 0,54 µm. Comme l'ont décrit de nombreux auteurs chez tous les vertébrés possédant des érythrocytes nucléés, la coloration vitale au bleu de crésyl brillant met en évidence dans le cytoplasme un matériel basophile granuleux. La conservation du noyau a comme corollaire la conservation d'une partie de l'ARN cytoplasmique. Cette substance basophile se présente comme la substance réticulo-filamenteuse des réticulocytes des mammifères.

4 % des cellules rouges du sang sont des proérythrocytes identiques aux proérythrocytes médullaires, et c'est dans le sang que peut être étudiée la maturation du proérythrocyte, c'est-à-dire sa transformation en érythrocyte. Cette maturation se caractérise à la fois par l'accroissement considérable du cytoplasme avec réduction du rapport N/P qui passe de 0,4 à 0,1, et par l'allongement de la cellule et du noyau. L'accroissement cytoplasmique est tel que la cellule grandit. Un stade intermédiaire est souvent observé : cytoplasme abondant et allongé avec noyau encore arrondi ou peu allongé.

À côté de la série proérythrocyte-érythrocyte telle qu'elle vient d'être décrite, on trouve dans le sang des cellules à noyau plus volumineux, dont les caractères morphologiques sont ceux des érythroblastes, ou se rapprochent de ceux des érythrocytes. Les premières sont incontestablement des érythroblastes, surtout du stade III (6,70 %), mais aussi du stade II (4 %) et rarement du stade I (1,4 %). Les secondes sont de grands érythrocytes (6,34 %) qui peuvent être considérés, en raison de la taille de leur noyau, comme dérivant directement sans mitose des érythroblastes III ou exceptionnellement des érythroblastes II. La maturation cytoplasmique se fait sans que le noyau ait été suffisamment réduit

de volume la dernière mitose (celle de l'erythroblaste III) et parfois l'avant-dernière (celle de l'erythroblaste II) sont sautées Ce processus normal chez le lézard, est celui de la mégaloblastose humaine arrêt des divisions érythroblastiques et maturation cytoplasmique

Resumé

Bien que les erythrocytes du lezard soient nettement differents de ceux des mammifères (persistance du noyau et d'un residu de substance basophile cytoplasmique) leur production est superposable à partir d'un proérythroblaste de grande taille quatre mitoses successives réduisent la taille nucléaire à $^1/_2$ $^1/_4$ $^1/_8$ et $^1/_{16}$ ce qui permet de différencier 5 types cellulaires Cependant le passage de la moelle dans le sang est moins sélectif que chez les mammifères on trouve dans le sang du lézard de nombreuses cellules qui n'ont pas subi toutes les mitoses et qui se transforment directement en érythrocytes plus grands et à noyau plus grand que la majorité des erythrocytes

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Demandes de tirés à part: Mme E. [illegible], Institut d'Hématologie, [illegible] Avenue [illegible] (France)

S. TURA and M. BACCARANI (eds.): Chronic Myeloid Leukaemia. Haematologica, Pavia 1972.

The book gives an excellent account of the most recent insights in the pathogenetic, cytogenetic, kinetic and immunological aspects of the disease. Therapeutic possibilities are thoroughly reviewed and new approaches are proposed. The book can be highly recommended to hematologists and oncologists.

B. SPECK, *Basel*

R. GROSS und J. VAN DE LOO (Hrsg.): Leukämie. Springer, Berlin 1972. 700 pp., 219 fig. DM 148.–

Das Buch gibt einen guten und sehr vielseitigen Überblick über den heutigen Stand in der Diagnostik und der Therapie der Leukämien.

Das Stück über die pathologische Anatomie ist sehr vollständig und gibt für den Kliniker wichtige Informationen über Todesursachen, Übergänge der einzelnen Leukämieformen, infektiöse und hämorrhagische Komplikationen. Ebenfalls sehr eindrücklich sind die zytochemischen und elektronenmikroskopischen Darstellungen. Zytogenetika, auch für die Frühdiagnostik und die Genetik der Leukämien, sind in einfacher und übersichtlicher Weise dargestellt. Die Dynamik und die kinetischen Aspekte der Leukämien werden in guter Form aufgeführt. Mehrere einschlägige Artikel behandeln die mehr konventionelle antileukämische Therapie, aber auch die Möglichkeit der Zellsynchronisation und der Immunotherapie werden *in extenso* behandelt. Das Stück über den Ersatz von korpuskulären Blutelementen ist adäquat, geht aber nicht sehr eingehend auf die modernsten Methoden des Granulozytenersatzes und der Knochenmarktransplantation ein. Die Behandlung von Leukämiepatienten in protektiver Isolation und ihre prospektive Bedeutung wird diskutiert. Dieses Buch ist meines Wissens zurzeit die umfangreichste deutschsprachige Darstellung des Problems der Leukämie. Es kann für hämatologisch und onkologisch tätige Ärzte und Bibliotheken sehr empfohlen werden.

B. SPECK, *Basel*

Table I. Incidence of pyroglobulins in our paraproteinemic patients

	Number of patients	Pyroglobulins
IgG Myeloma	151	1
IgA Myeloma	51	1
IgD Myeloma	2	
Bence Jones myeloma	12	
Waldenström's disease without cryoglobulinemia	39	3
Waldenström's disease with cryoglobulinemia	5	3
Total	2[illegible]0	8

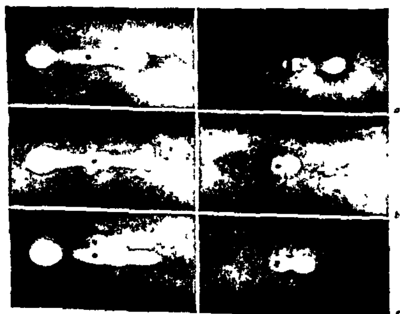


Fig. 1. [illegible] of 3 pyroglobulinemic sera. On the left the slides were [illegible], on the right [illegible] heated to 60 °C [illegible]. *a* Case M. L., IgG myeloma. *b* Case I. B., Waldenström's disease. *c* Case M. A., IgA myeloma.

Materials and Methods

The presence of pyroglobulins was tested for by warming up to 56–60 °C of the serum taken from our paraproteinemic patients. Zone electrophoresis of serum was performed in agar gel using Agar Noble (Difco) 2% at pH 8.2 in 0.05 ionic strength barbital buffer or in cellulose acetate membrane using pH 8.6 barbital buffer solution of 0.075 ionic strength. Immunoelectrophoresis was performed according to SCHEIDEGGER [11]. Analytical ultracentrifugation was performed by means of a Spinco Model E centrifuge at 52 000 rpm, 27 °C.

Isolation of pyroglobulins. Purified preparations were obtained by preparative electrophoresis in agar gel or in starch block. The γ globulins collected were separated by passing them through Sephadex G 200 (2.5 × 100 cm. Pharmacia) column using 1 M sodium chloride in 0.1 M tris HCl, pH 8, or through a DEAE column starting buffer of 0.01 M phosphate, pH 8, final buffer of 0.3 M phosphate, pH 8.

Effects of chemical reagents on thermoprecipitability. Reduction and alkylation of serum or of isolated proteins were carried out with 0.1 M 2 mercaptoethanol and with dialysis against 0.02 M iodacetamide. Other effects on thermoprecipitability were ascertained using dialysis against hypertonic solutions of NaCl (from 0.9 to 3%) or against phosphate buffers at pH ranging from 3 to 7.

Results

Characteristics of Eight Pyroglobulinemic Serums

The distribution of pyroglobulins among our paraproteinemic patients is summarized in table I. As can be seen, pyroglobulins are much more frequent in Waldenström's disease (6 cases out of 44), especially when associated with cryoglobulinemia (3 cases out of 5), than they are in myeloma (2 out of 216).

Of particular interest are the findings with regard to a case of IgA myeloma: only one case of an IgA pyroglobulin has been previously reported [12]. In table II, some clinical and serological data of our cases are given. The high sedimentation constant of cases S. N., P. E., C. A. is due to the associated mixed cryoglobulins, which results in circulating IgM-IgG complexes.

Pyroglobulin Characterization

(1) Analysis of whole serum. In all our cases, there was electrophoretical identity between pyroglobulin and paraprotein (fig. 1). This identity was confirmed by the loss of M-component from serum after precipitation of pyroglobulin (fig. 2).

(2) Analysis of isolated pyroglobulins. The electrophoretic, immunoelectrophoretic and ultracentrifugal characteristics of an IgG and IgA

Table I. Incidence of pyroglobulins in our paraproteinemic patients

	Number of patients	Pyroglobulins
IgG Myeloma	153	1
IgA Myeloma	51	1
IgD-Myeloma	2	
Bence Jones myeloma	12	
Waldenström's disease without cryoglobulinemia	39	3
Waldenström's disease with cryoglobulinemia	5	3
Total	260	8

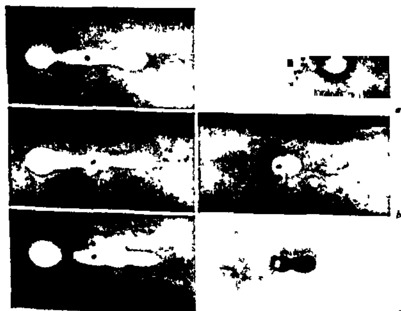


Fig. 1. Agar electrophoresis of 3 pyroglobulin sera. On the left, the slides were fixed in [illegible] and on the right identical preparation heated to 60 °C without fixing. a Case M. L., IgG-myeloma. b Case L. R., Waldenström's disease. c Case M. S., IgA-myeloma.

Table II Summary of clinical and

Patient	Sex	Age	Diagnosis	Cryoglobulins (cryocrit)	Free serum L-chain	Urine proteins B J
M L.	M	59	myeloma	–	–	+
I R	M	55	macroglobulinemia	–	–	–
F A	M	60	macroglobulinemia	–		–
S N	F	61	macroglobulinemia	IgM IgG (20%)	–	–
D T L.	F	66	macroglobulinemia	–	–	–
P E	F	44	macroglobulinemia	IgM IgG (15%)		–
C A	F	46	macroglobulinemia	IgM IgG (27%)	λ	+
M A	F	58	myeloma	–	λ	+

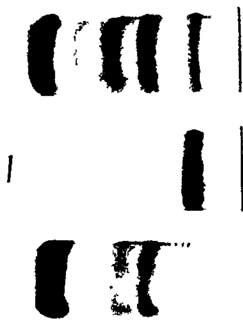


Fig 2 Cellulose acetate electrophoresis of serum M L. (IgG myeloma) Whole serum (upper) the isolated M-component (middle) the supernatant after heating serum at 48 °C (lower)

isolated pyroglobulins are shown in figures 3 and 4 As to the cases with associated cryoglobulinemia we found that pyroglobulins were recovered almost completely, in the redissolved cryoprecipitate The isolation of the 2 components, IgM and IgG of the mixed cryoglobulins showed that pyroprecipitation depends exclusively on the IgM-component (fig 5)

[illegible] features of patients

[illegible]	Total proteins g%	M-Components			
		electrophoresis %	heavy chain class	light chain type	sedimentation constant (S 20 W)
	11	γ_1 63	IgG	κ	7
	11.4	γ_1 61	IgM	κ	19
	10	γ_1 44	IgM	λ	19
•	19	γ_1 52	IgM	κ	21
	8.7	γ_1 26	IgM	κ	19
	8.4	γ_1 37	IgM	κ	23
•	10.8	γ_1 44	IgM	κ	23
•	9	β 30	IgA	κ	12.3

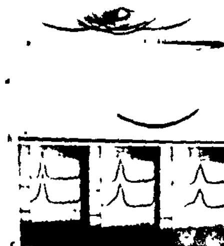


Fig. 3. Case M. M. (IgG myeloma). a Immunoelectrophoresis [illegible] serum (upper [illegible]) and isolated M-component (lower well): precipitation [illegible] with rabbit [illegible] serum. b Immunoelectrophoresis of isolated M [illegible] in the [illegible] serum. c Analytical ul[illegible] photo[illegible] taken at 24, 32, 64 min, respectively, after reaching [illegible] sedimenta[illegible] [illegible] Upper: isolated M-component. Lower [illegible]

(3) Effect of various reagents on pyroprecipitation [illegible] such [illegible] are presented in table III. The treatment [illegible] ethanol [illegible] and acetamide did not inhibit the heat [illegible] Lower[illegible] pH in most cases increase [illegible] precipita[illegible] [illegible]

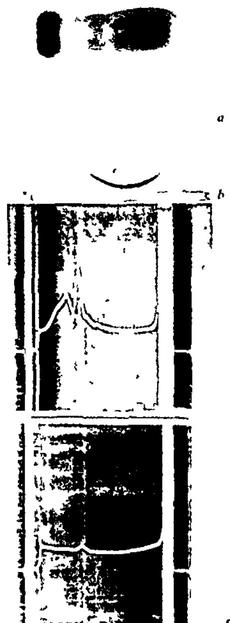


Fig. 4. Case M. A. (IgA myeloma). *a* Cellulose acetate electrophoresis of serum and isolated M-component. *b* Immunoelectrophoresis of isolated IgA paraprotein in the trough; rabbit anti immunoglobulin serum. *c* Ultracentrifuge pattern. Upper: whole serum; lower: isolated IgA. Photographs taken at 32 min

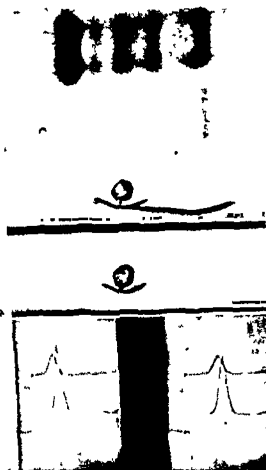


Fig. 3. Case S. N. (Waldenström's disease with cryoglobulinemia). a Cellulose acetate electrophoresis of serum (upper) and isolated paraprotein (lower). b Immunoelectrophoresis: in the upper well the isolated components (IgM, IgG) of the cryoprecipitate, in the lower the purified IgM. In the trough an immunoglobulin antiserum. c Analytical ultracentrifugation of isolated IgM. Photographs taken at [illegible] and 24 min after reaching 52,000 rpm.

due to a breaking of H-ion bonds effected by the acidity [illegible] favouring the successive action of heating. Only in a serum with IgM pyroglobulin we observed, at the same conditions, a delay in thermo-[illegible] [illegible]. We do not know the cause of this variability.

Table III Effect of various conditions on precipitability of pyroglobulins at 60 C

Patient	Pyroglobulin	2 ME	pH 4	3% NaCl
M L	IgG κ	0	0	–
I R	IgM κ	0	–	–
I A	IgM λ	0		0
S N	IgM κ	0	+	–
D T L	IgM κ	0	+	+
P E	IgM κ	0	0	
C A	IgM κ	0	+	–
M A	IgA κ	0	+	–

0 = No effect, + = increased pyroprecipitation – = partial inhibition of pyroprecipitation

On the other hand, dyalisis against hypertonic solutions partially inhibited the pyroprecipitation the higher activity of hydrophobic groups in hypertonic solutions resulting in a greater stability of the molecule can explain this finding

Discussion

Our results confirm previous reports on the close association between pyroglobulinemia and paraproteinemia The incidence of this finding is about 2–3% (4 cases out of 162 in the series of COLLIER *et al* [2], 8 out of 260 in ours), conversely we have found no cases without paraproteinemia A possible explanation of the association is that pyroglobulins normally present in serum in very low concentration, reach detectable serum levels only when an immunoproliferative disease affects the clone specifically committed to their synthesis, on the contrary can be conceived pyroglobulins as abnormal immunoglobulins absent in normal serum A response to this question *is strictly connected with the answer about the nature* of the M-components

Another interesting finding is the high frequency of the association between pyroglobulins and mixed cryoglobulins in Waldenstrom s disease (3 pyroglobulins out of 5 cases with cryoglobulins on a total of 44 cases of Waldenström s disease) These findings already reported by MELTZER and FRANKLIN [6] (1 case) are particularly evident in our report

In the present study we have demonstrated the identity of the molecule IgM responsible for the 2 phenomena. In both cases, only H-chains are engaged in the thermal reactions [1, 8]. Contrary to the results of PATTERSON *et al.* [10], regarding the presence of both IgM and IgG in an IgM pyroglobulin precipitate dissolved in 2-mercaptoethanol, we have found that only the IgM, isolated from a mixed IgM-IgG-cryoprecipitate, was the pyroprecipitable component.

Moreover, we have investigated the molecular basis of pyroprecipitation. The different response from each pyroglobulin to the same physicochemical agents does not support any conclusion. This variability is probably due to the various effects of thermal denaturation, giving rise sometimes to intermolecular complexes, at times active only inside the molecule.

PATTERSON *et al.* [10] arrived at the same conclusion by observing a variable pattern in thermoprecipitation not only between pyroglobulins of different classes (IgM and IgG), but also of the same class (IgG). MORST [7] studying the susceptibility to heat aggregation of isolated myeloma globulins, showed that 11 of 18 failed to aggregate independently of their electrophoretic mobility, thus suggesting a great variability in the degree of thermoreactivity between immunoglobulins. According to ZINNEMAN and SEAL [13], the possibility of bringing into solution the pyroprecipitate by sodium dodecyl sulfate, an amphiphile, suggests that hydrophobic bonding are implicated in the mechanism of thermoprecipitation.

In our cases, the thermolability was not inhibited by 2-mercaptoethanol followed by iodoacetamide; similar result was observed by SUGAI [12] in his case of IgA pyroglobulin. This indicates that the formation of intermolecular disulfide bonds is not the only mechanism of precipitation.

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D T L	IgM κ	0	+	+
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P F	IgM κ	0	0	
C A	IgM κ	0	+	–
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Acta Haemat. [illegible]: 75–84 (1973)

Viskositätsuntersuchungen mit Fibrinogenspaltprodukten

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Abstract. In in vitro investigations using the Hark ness viscosimeter a logarithmic increase of the viscosity could be demonstrated for increasing concentrations of fibrinogen degradation products (FDP). This increase of viscosity could also be observed with increasing concentrations of FDP plus fibrinogen, however, increasing concentrations of fibrinogen showed a linear increase of viscosity. Increasing concentrations of FDP plus globulins showed a linear curve in contrast to the exponential increase of viscosity of fibrinogen plus globulins.

Additionally, FDP showed a lower viscosity in contrast to fibrinogen of a comparable viscosity using a Brookfield viscosimeter at different shear rates in presence of a suspension of erythrocytes.

Key Words
Fibrinogen degradation
Fibrinolysis
Viscosity

Als Faktoren der intravasalen Mikrozirkulation gelten neben Perfusionsdruck und Gefässdurchmesser die Blut- und Plasmaviskosität, Aggregationstendenz und Formveränderung der Erythrozyten, Thrombozytenaggregation und Gerinnungsvorgänge wie Verbrauchskoagulopathie und Fibrinolyse [4–10]. Störungen der Mikrozirkulation hängen daher eng von Veränderungen dieser Parameter ab.

Genauere Untersuchungen über den Zusammenhang und die Abhängigkeit der Blut- und Plasmaviskosität von Fibrinogenspaltprodukten (FDP) wurden bisher nicht durchgeführt.

[1] Wir danken der [illegible] für [illegible]

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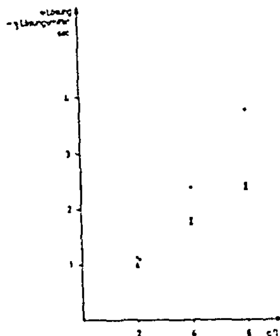


Abb. 2 [illegible] verhalten des Fibrinogens nach Fibrinogenolyse bei unterschiedlichen [illegible] = Fibrinogenlösung + Streptokinase [illegible] + [illegible] ACA (0,6 mg/ml) [illegible] = Fibrinogenlösung + Streptokinase (100 [illegible]) + Trasylol (500 [illegible])

wurde AG [illegible] und Aprotinin (Trasylol® Bayer, Leverkusen) durchgeführt. [illegible] der Viskosität verlauf und Viskositätszahl [illegible] Fibrinogenlösung [illegible] (2, 4, 6 g/l) nach Zugabe [illegible] Streptokinase [illegible] (Abb. 1) und nach [illegible] Inkubation bei 37 °C [illegible] Hemmung der Fibrinogenolyse mit EACA (0,6 mg [illegible]) und Trasylol (500 [illegible]) gemessen (Abb. 2).

[illegible] Versuch [illegible] EACA (0,6 und 1,2 mg/ml) oder Trasylol [illegible] und [illegible] Streptokinase (100 [illegible]) gegeben (Abb. 3) und nach Inkubation [illegible] der [illegible] Vergleich [illegible] Fibrinogen [illegible] gemessen. [illegible]

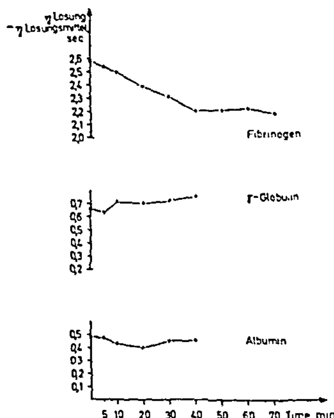


Abb. 1 Die Einwirkung von Streptokinase (je 100 E ml) auf Fibrinogen γ-Globulin und Albumin

Material und Methodik

Die Viskosität der Fibrinogen γ-Globulin und FDP Lösungen wurden mit dem Harkness Viskosimeter [5] gemessen das einen konstant reproduzierbaren Schergrad von 660 sec⁻¹ einen konstanten Druck von 75 mm Hg und eine kinematische Viskosität von 2 10⁻² cm² sec hat Die Ablesung erfolgte durch elektrische Zeitmessung mit einer Genauigkeit von 0 02 sec als Durchschnittswert von 5 Einzelmessungen Die Angabe der Viskosität erfolgte in Sekunden als relativer Wert zum Lösungsmittel (η Lösung η 0 9% ige NaCl) Die Viskosität der Erythrozytensuspension wurde mit einem Wells Brookfield Viskosimeter Modell LVT, bei den Schergraden 115 46, 23 und 11,5 sec⁻¹ bei 37 C gemessen [12] Begonnen wurde beim Schergrad 115 sec⁻¹ gesichert wurde fur zirka 2–3 min und zwischen jeder Messung die Suspension durch Kippen des Tellers neu vermischt

Die Untersuchungen wurden mit Fibrinogen (Forschungs Fibrinogen Kabi Deutsche Kabi GmbH München 15) γ-Globulin (γ-Globulin aus Humanserum lyophil, rein 95% Behringwerke AG Marburg Lahn) sowie Streptokinase (Streptase® Behringwerke AG Marburg Lahn) ε Aminocapronsäure (EACA Behring

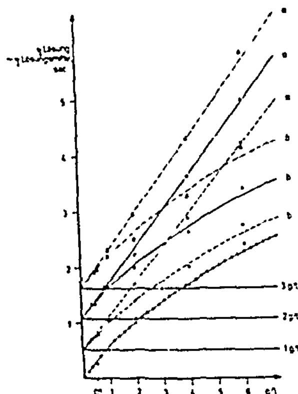


Abb. 4. Viskositätsverhalten von FDP und Fibrinogen im Vergleich zu ansteigenden Fibrinogenkonzentrationen in einem Konstant-Fibrinogen (1, 2 und 3 g/l). Variabel: a = Fibrinogen (0,5, 1, 2, 4 und 6 g/l), b = FDP sowie + + + + + = FDP als Einzelmessung (b und • • • • • a = 0,5, 1, 2, 4 und 6 g/l Fibrinogen nach Streptokinaseinkubation mit 100 E/ml 60 min)

über etwa 40 min ab. Nach dieser Zeit konnte keine weitere Viskositätsänderung gemessen werden (Abb. 1). Albumin und γ-Globuline zeigten unter Streptokinaseeinwirkung keine Viskositätsänderung (Abb. 1).

Der Viskositätsrückgang beim Fibrinogen unter Streptokinaseeinwirkung hängt von der Ausgangskonzentration und Ausgangsviskosität ab und ist um so ausgeprägter, je höher diese anfangs waren (Abb. 2). Die gleichzeitige Gabe von EACA + Streptokinase zu Fibrinogen verhindert den bei alleiniger Gabe von Streptokinase beobachteten Rückgang der Viskosität [illegible] während 40 min nicht (Abb. 2 und 3). Die gleichzeitige Ga

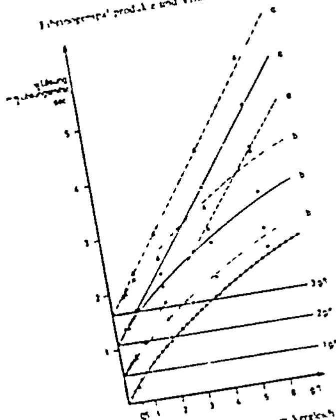


Abb. 4. Viskositätsverhalten von FDP und Fibrinogen im Vergleich zu ansteigenden Fibrinogenkonzentrationen [illegible] Konstant Fibrinogen (1, 2 und 3 g/l) Variabel [illegible] gen (0,5; 1; 2; 4 und 6 g/l). [illegible] FDP [illegible] = FDP [illegible] (0,5; 1; 2; 4 und 6 g/l) Fibrinogen nach [illegible] mit 100 [illegible]

über etwa 4 Stunden ab. Nach dieser Zeit konnte keine weitere Viskositätsänderung gemessen werden (Abb. 1). Albumin und γ-Globuline zeigten unter Streptokinaseeinwirkung keine Viskositätsänderung (Abb. 1).

Der Viskositätsrückgang beim Fibrinogen unter Streptokinaseeinwirkung [illegible] hängt von der Ausgangskonzentration und Ausgangsviskosität ab [illegible] je höher diese anfangs waren (Abb. 2). Die [illegible] Gabe von EACA [illegible] Streptokinase zu Fibrinogen [illegible] Rückgang der [illegible] Gabe von Streptokinase beobachteten Rückgang der Viskosität während 4 Stunden nicht (Abb. 2 und 3). Die gleichzeitige Gabe [illegible]

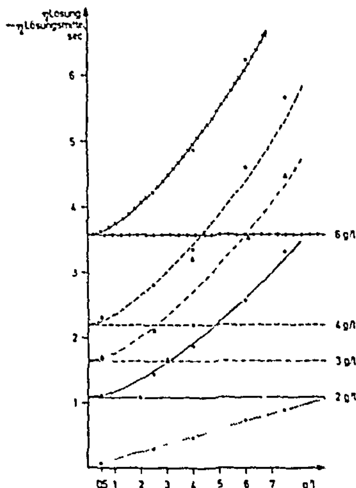


Abb 5 Viskositätsverhalten von Fibrinogen und γ-Globulinen in unterschiedlichen Konzentrationen Konstant Fibrinogen —+—=2 g/l, ---▲---=3 g/l, -- -●- =4 g/l, ···×···=6 g/l Variabel γ-Globulin (0,5, 2,5, 4, 6 und 7,5 g/l), + =γ-Globulin (Einzelmessung)

von Trasylol + Streptokinase zu Fibrinogen bewirkt dagegen keine Viskositätsänderung Eine Hemmung der Streptokinaseeinwirkung lässt sich daher *in vitro* nur durch Trasylol, nicht durch EACA durchführen (Abb 3) Eine Eigenwirkung des Trasylols bzw der EACA auf die Viskosität kann ausgeschlossen werden (Abb 3)

Der Viskositätsverlauf unterschiedlicher Fibrinogenkonzentrationen zeigt ein additives Verhalten und einen linearen Verlauf (Abb 4) Der Viskositätsverlauf einer FDP-Lösung aus unterschiedlichen Konzentratio-

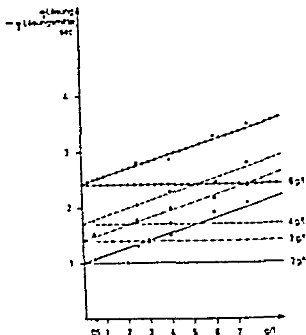


Abb. 6. Viskositätsverhalten von FDP und γ-Globulinen unterschiedlicher Konzentrationen. Konstant: FDP (aus 2, 3, 4 und 6 g/l Fibrinogen nach Streptokinaseinkubation mit 100 E/ml 60 min) = ——— = 2 g/l, – –▲– – = 3 g/l, – –●– – = 4 g/l, + + + + + = 6 g/l. Variabel: γ-Globulin (0,5; 2,5; 4; 6 und 7,5 g/l)

ten einer Fibrinogenlösung durch Streptokinase hergestellt, zeigt einen logarithmischen Verlauf (Abb. 4). Der Viskositätsverlauf unterschiedlicher Fibrinogenkonzentrationen mit unterschiedlichen FDP zeigt ebenfalls ein additives Verhalten, jedoch einen logarithmischen Kurvenverlauf (Abb. 4). Der Viskositätsverlauf unterschiedlicher Fibrinogenkonzentrationen mit unterschiedlichen γ-Globulinkonzentrationen zeigt im Gegensatz zu reinem γ-Globulin (Abb. 5) und reinem Fibrinogen (Abb. 4) ein additives Verhalten und einen exponentiellen Verlauf (Abb. 5). Der Viskositätsverlauf unterschiedlicher FDP-Konzentrationen mit unterschiedlichen γ-Globulinkonzentrationen zeigt ebenfalls ein additives Verhalten und [illegible] einen linearen Verlauf (Abb. 6). Eine Erythrozytensuspension, der FDP oder Fibrinogen gleicher Viskosität zugegeben wurde, zeigt mit FDP bei allen Schergraden eine niedrigere Viskosität (Abb. 7).

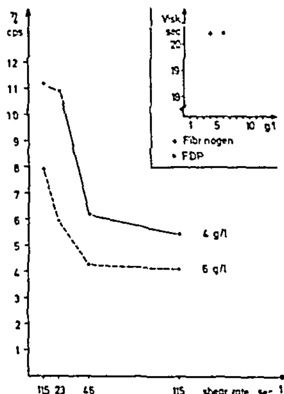


Abb. 7. Verlauf der Viskosität einer Erythrozytensuspensionslösung, der Fibrinogen oder FDP gleicher Viskosität zugegeben wurden. ——+—— = 4 g/l Fibrinogenlösung mit Erythrozytensuspension (Hämatokrit 40 ± 1%, 37°C). --+--- = 6 g/l FDP-Lösung nach Streptokinaseinkubation (mit 100 E/ml, 60 min) mit Erythrozytensuspension (Hämatokrit 40 ± 1%, 37°C)

Diskussion

Viskositätswirksame Parameter für die Plasmaviskosität sind das Fibrinogen und die γ-Globuline und für die Vollblutviskosität zusätzlich der Hämatokrit und die Erythrozytenaggregationstendenz [7]. Besonders Wechselwirkungen des Fibrinogens mit den γ-Globulinen und den Erythrozyten sind hierfür verantwortlich, und für Veränderungen der Viskosität werden daher vorwiegend Konzentrationsänderungen des Fibrinogens angeführt [3, 8]. Besonders eindrucksvoll konnte dies an therapeutischen Fibrinolysen, die mit einer Fibrinogenolyse einhergehen, demonstriert werden [1, 2, 4].

Trotzdem sollten die bei unterschiedlichen Krankheiten und besonders bei fibrinolytischen Zuständen anfallenden FDP [6] hinsichtlich ihrer Vis-

kositätswirksamkeit untersucht werden. Dabei konnten wir aufgrund unserer Untersuchungen einen wesentlich geringeren viskositätswirksamen Effekt der bei einer Fibrinogenolyse aus dem hochmolekularen Fibrinogen entstehenden niedermolekularen Spaltprodukte D und E [11] sowohl auf das Fibrinogen und die γ-Globuline als auch auf eine Erythrozytensuspension feststellen. Klinisch bedeutet dies, dass bei Krankheitsbildern, die mit dem Auftreten besonders hoher Konzentrationen von FDP einhergehen [6], die Plasmaviskosität sowie ihr Einfluss auf die Vollblutviskosität sich anders verhält als bei Krankheiten ohne FDP, aber etwa gleicher Fibrinogen-, γ-Globulin- und Hämatokritkonzentration.

Zusammenfassung

In den Viskositätsuntersuchungen mit dem Harkness-Viskosimeter konnte für ansteigende Konzentrationen von Fibrinogenspaltprodukten (FDP) ein logarithmischer Viskositätsanstieg gezeigt werden. Dieser logarithmische Viskositätsanstieg trat ebenfalls bei ansteigenden Konzentrationen von FDP plus Fibrinogen auf, während ansteigende Fibrinogenkonzentrationen allein einen linearen Viskositätsanstieg zeigten. Ansteigende Konzentrationen von FDP plus γ-Globulinen zeigten im Gegensatz zum exponentiellen Viskositätsanstieg von Fibrinogen plus γ-Globulin einen linearen Verlauf. Ausserdem zeigten am Brookfield-Viskosimeter bei unterschiedlichen Schergraden FDP im Gegensatz zum Fibrinogen gleicher Ausgangsviskosität mit einer Erythrozytensuspension eine niedrigere Viskosität.

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Acta Haemat. 50: 85–91 (1973)

Binding of Folic Acid to Serum Proteins

I. The Effect of Pregnancy

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Abstract. Binding of serum folic acid activity (FAA) to the protein fractions during and after pregnancy was studied by DEAE Sephadex A-50 gel chromatography. Towards the end of pregnancy this binding increased. The increase was particularly distinct in the binding to transferrin. At the same time the binding to α2-macroglobulin decreased. Within a week after delivery, the total protein binding of FAA decreased remarkably. The decrease was also particularly distinct for transferrin.

Key Words
Carrier proteins of folic acid
Folic acid metabolism
Gel chromatography
Pregnancy
Serum proteins

In our laboratory, it could be shown that just under half of the serum folic acid activity (FAA) is bound to various proteins [5–7, 9]. Some FAA travel with albumin [5], a finding that has been corroborated elsewhere [2]. Usually, the majority of the bound FAA is filtered in chromatography with α2-macroglobulin [10] and the balance with transferrin [11]. These results have been obtained from fresh blood specimens of healthy test subjects. Owing to bacterial contamination, however, the chromatographies had to be carried out in cold conditions, at +10 °C, under ... light, and it is naturally possible that temperature might affect the binding of FAA with proteins. In the course of a fairly long follow-up period, the chromatography results obtained from one and the same person has always been very much the same (unpublished observation).

Since the normal chromatographic distributions of FAA have now been clarified in this way, it is interesting to study how the different factors – physiological or pathological – affect of normal FAA distribution. Pregnancy is known to be a factor which considerably changes the total

homeostasis of serum FAA Some results of a preliminary study of the influence of pregnancy on the binding of FAA to serum proteins are presented in the following

Material and Methods

The changes in the binding of serum FAA during and after pregnancy were followed in 2 primiparas by chromatography Detailed characteristics can be seen from table I The examined women were in good health and ingested Finnish staple food without additives throughout the period of observation For the present study they attended a special examination and underwent laboratory tests after the 20th (chromatography A) and the 30th (chromatography B) week of gestation and again 5 days (chromatography C) and 8 weeks (chromatography D) after normal delivery

Chromatographies were made on absolutely fresh venous blood specimens The treatment of the specimens and the chromatography techniques have been described in detail before [10] Protein determinations were carried out using the biuret reaction and the FAA determinations with the L. casei method in the way described earlier [10]

Results

Figure 1 presents the chromatogram of a non-pregnant healthy young woman's serum FAA is hardly seen at all in the first protein peak (γ-globulin) The second peak (γ-globulin and transferrin) shows 22% of the bound FAA, the third (the flat intermediate zone, transferrin) 10% and the fourth peak (α-2-macroglobulin) 30% of the bound FAA The remainder of the FAA is scattered over the albumin area Of the total FAA of the serum, 26% is bound to the protein area

A review of both young women's chromatograms during pregnancy (fig 2–3) shows that the A chromatogram (20th week of gestation) is very similar to that of a non pregnant woman Table I reveals, however that the FAA binding percentage to serum proteins in the pregnant woman already is increased in this stage The B chromatograms of the pregnant woman (30th week of gestation) disclose a violent increase in FAA binding for in both test persons almost all serum FAA is bound to proteins, and the binding rate per protein unit has doubled (table I) The FAA increase in the transferrin area is particularly conspicuous patient T V showed an increase from 45 to 50% and patient P T from 45 to 78%



Fig. 1. Chromatogram of the serum of a healthy non-pregnant woman. Vertical lining indicates FAA against the background of the protein chromatogram, in which the first peak contains γ-globulin, the second β-globulin and transferrin, the third (that intermediate area) transferrin, the fourth α2-macroglobulin, and the fifth [illegible] albumin. The total FAA in the serum is 4700 pg/ml, of which 29% are bound to proteins.

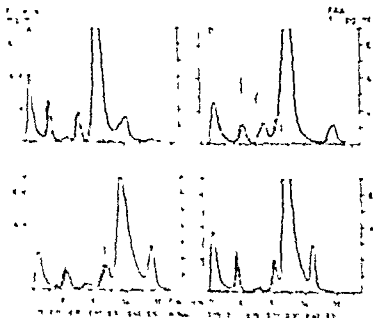


Fig. 2. The pregnant test subject T.V. For details see table I. Chromatograms A and B during pregnancy, C and D after delivery. Note the increased binding of FAA in [illegible] B to the second and third protein peaks.

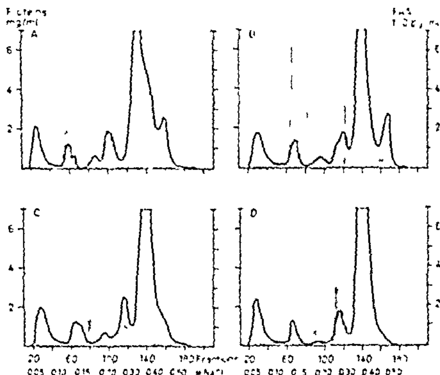


Fig. 3. The pregnant test subject P. T. For details, see table 1. As in figure 2 chromatogram B reveals a distinct increase in FAA in the area of the second and third protein node. Chromatogram C shows the disappearance of FAA from the corresponding areas.

The amount of FAA bound to α-2-macroglobulin has in both cases fallen by 15–20% compared with the earlier date of pregnancy (chromatography A).

Figures 2 and 3 and table 1 reveal, further, that a heavy fall has taken place in the protein binding of FAA immediately after delivery (chromatography C). The fall took place mainly in the transferrin area, but some FAA has disappeared also from the albumin area. The change can be considered the exact opposite of the one taking place as pregnancy advanced. The fall in FAA binding was particularly violent in test subject P. T., not less than from 61 to 17 ng/g (table 1). At the same time, it could be ascertained that no essential changes occurred in the protein content of the serum around the time of delivery. Also, the total FAA of the serum had remained approximately on the level recorded during pregnancy.

Table I. Detailed information on 2 pregnant test subjects at each chromatography[1]

Test person	Time of chromatography	Hb g%	Weight kg	Total FAA ng/ml	Serum protein mg/ml	FAA protein-bound ng	FAA protein-bound percentage
P.T.							
	A	10.5	60.2	3.9	65.6	30.4	47
	B	10.5	62.9	4.0	61.6	41.2	92
Delivery after 37 weeks of pregnancy							
	C	12.1	57.5	2.7	64.8	17.2	41
	D	12.0	41.5	1.8	66.4	26.1	92
T.V.							
	A	11.7	63.7	2.7	65.6	31.6	75
	B	12.0	73.0	4.0	64.4	65.1	100
Delivery after 35 weeks of pregnancy							
	D	12.3	45.5	2.7	61.2	34.1	74
	C	11.[illegible]	55.0	3.1	67.6	25.0	52

[1] A after 20, B after 30 weeks of gestation, C 5 days and D 8 weeks after delivery. Test subject P.T. was 20 years old, and the child's birth weight was 2,450 g. Test subject T.V. was 23 years old and the child's birth weight was 2,620 g.

The chromatographs C figures (8 weeks after delivery) show that the later phase of lactation the protein binding of FAA begins to resem[ble] the normal proportions. The binding in this phase perhaps still excee[ds] that in a non-pregnant woman. During lactation, test subject P.T. (fig. 3) developed a distinct FAA deficiency and almost all of her serum FAA w[as] therefore bound to protein. In this phase of lactation no bound FAA at all is found in the albumin area. This was never the case with the non-pregnant woman nor with either of the test subjects in the different phases of pregnancy.

Discussion

On the basis of this preliminary study, we may draw the conclusion that the binding of FAA to proteins in the maternal serum increases during pregnancy. A particularly striking increase takes place in the transfer[illegible]. After delivery this accumulation disperses, and at the same time the FAA bound to albumin again appears.

On the basis of our earlier studies, we have reported that in a healthy human individual the serum FAA, examined by chromatography, is eluted from the column carried by albumin, α-2-macroglobulin and transferrin [8, 10 11] Very infrequently FAA is seen to be bound to γ-globulin (the first protein peak in DEAE Sephadex chromatography) [our unpublished finding] Naturally, this gives rise to the question as to why FAA should have so many binders or carrier proteins The kinetics of FAA have not as yet been extensively studied The series of studies now inaugurated will, it is hoped, provide some tentative suggestions in this problem

According to the preliminary studies, chromatography carried out under identical experimental conditions and in particular, in exactly the same way, always gives the same result for any one test person, both as regards the distribution of proteins and that of FAA [our unpublished finding] The repeatability of the chromatography result is in our opinion extremely good Hence, the change in FAA binding during pregnancy may probably be considered reliable So far the physiological role of the change described is not known, nor are the factors that produce it known As pregnancy advances, the protein content of blood per volume unit decreases, with the result that albumin decreases, α-1-, α-2-macroglobulin and transferrin increase, and β-2- and γ-globulin remain practically constant [12] Similar changes were also observable in the present study, however, the relative increase in FAA per protein unit was very much more definite It seems that, towards the end of pregnancy, FAA accumulates especially in transferrin from which it disperses immediately after delivery (into the mother's milk?) At the same time, the FAA which has been bound in the albumin area disappears For α-2-macroglobulin, no particularly pronounced changes seem to take place either way It might be assumed that albumin and transferrin are, in FAA metabolism, first degree carrier proteins and that the α-2-macroglobulin is a second-degree carrier which retains the FAA stores to the very last moment In this connection it should also be noted that the above 2 components (albumin and transferrin) are synthesized in the liver

According to a great deal of the literature, serum FAA decreases during pregnancy, and this may lead to the development of a deficiency state On the basis of the present study it may be deduced that much greater changes perhaps take place in the internal distribution of serum FAA than in the total serum FAA For this reason the aim of future research should, first and foremost be to study the possible physiological factors

which produce the change in the distribution (the roles of the various hormones, liver, etc.) [1, 3, 4] and the reasons why such a change takes place towards the end of pregnancy. Does it ensure an adequate FAA content of mother's milk? Does it retain FAA for the mother's haematopoietic needs? Or are there several simultaneous contributory factors? In any case, it is apparent that the binding of FAA to serum proteins decreases relatively soon after the delivery. The preliminary studies have shown that the prolonged use of hormonal contraceptives produces the changes described here as having taken place during pregnancy [our unpublished finding].

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Acta Haemat. 50: 92–104 (1973)

Spezifische Mikrogranula in Eosinophilen

Eine vergleichende elektronenmikroskopische Untersuchung an verschiedenen Säugern zur Charakterisierung einer besonderen Granulationsform bei eosinophilen Granulozyten[1]

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Abstract. Besides the well known eosinophilic granules, mature eosinophil granulocytes are containing a special form of smaller granules measuring about 100 (20–200) nm. The constant occurrence of these *specific microgranules (SM) of eosinophils*, as they are named, is proved in eosinophils of 20 different mammalian species out of 7 orders including man. SM are composed of an opaque core bound with an unit membrane. SM may be distinguished into 3 varieties, a spheric (double spheric), a dumbbell, and a cup type. The occurrence of one or the other type depends on the species. SM are supposed to be an essential cytoplasmic detail of mature eosinophils hitherto widely disregarded.

Key Words
Comparative haematology
Electron microscopy
Eosinophil granulocytes
Specific microgranules
Ultrastructure of eosinophils

Bei früheren Untersuchungen [21] zur Alterung eosinophiler Granulozyten fielen in reifen Eosinophilen der Ratte elektronenmikroskopisch runde, granulumartige Gebilde auf, die sich von den bekannten eosinophilen Granula durch abweichende Struktur und weit geringere Dimension unterschieden. Solche Körper sind in der bisherigen Literatur zur Ultrastruktur der Eosinophilen weitgehend unbeachtet geblieben oder nur sporadisch als grössenordnungsmässig etwa 100 nm messende Gebilde in Eosinophilen einiger Laboratoriumstiere erwähnt worden [11, 16, 17, 19, 24]. Unsere Untersuchung hat zum Ziel, an einem möglichst breiten Spektrum verschiedener Säugerarten zu prüfen, ob solche Mikrogranula als regelmässiges und obligates Element der zytoplasmatischen Differenzierung reifer eosinophiler Granulozyten aufgefasst werden können, ob

[1] Mit freundlicher Unterstützung der Deutschen Forschungsgemeinschaft.

also die von uns verwendete Arbeitsbezeichnung „spezifische Mikrogranulas" [22] gerechtfertigt ist.

Material und Methodik

Leukozyten folgender 20 Spezies (systematische Bezeichnung und Anzahl untersuchter Exemplare in Klammern) wurden untersucht: 1. Ameisen- oder Zwergbeutelratte (*Marmosa murina*, 5), 2. westeuropäischer Igel (*Erinaceus europaeus*, 2), 3. Ohrenigel (*Hemiechinus auritus*, 3), 4. Eichhörnchen (*Sciurus vulgaris*, 2), 5. Bisamratte (*Ondatra zibethica*, 2), 6. Schermaus (*Arvicola terrestris*, 5), 7. Zwergmaus (*Micromys minutus*, 3), 8. Wanderratte (*Rattus norvegicus*, 20), 9. zentralafrikanische Mausart von der Insel Fernando Po (*Praomys morio*, 7), 10. zentralafrikanische Mausart aus der Region des Kamerunberges (*Praomys* [illegible], 3), 11. palästinensische Stachelmaus (*Acomys cahirinus*, 5), 12. kretische Stachelmaus (*Acomys minous*, 5), 13. Meerschweinchen (*Cavia porcellus*, 7), 14. Sumpfbiber (*Myocastor coypus*, 1), 15. Haushund (*Canis familiaris*, 3), 16. Hauskatze (*Felis catus*, 2), 17. Tiger (*Panthera tigris*, 2), 18. Wildkaninchen (*Oryctolagus cuniculus*, 3), 19. Hauspferd, Kaltblut (*Equus caballus*, 3) und 20. Mensch (*Homo sapiens*: 5 Gesunde, je 1 Fall von chronischer Myelose und akuter Myelose [illegible]).

Bei den Kleinsäugern wurde das Blut mit 1%igem Natriumzitrat versetzt bei der Tötung in Äthernarkose durch Herzpunktion, bei Hund, Tiger und Katze durch Punktion der Vena saphena parva, beim Pferd durch Punktion der Vena jugularis und beim Menschen aus der Kubitalvene gewonnen. Anschließende Zentrifugierung erfolgte 10 min bei 1500 Umdrehungen/min (Rotordurchmesser 13 cm). Versuchsgemäß Überschichtung der Leukozytenschicht mit wässriger Glutaraldehydlösung (6,25%ig in 0,1 m Phosphatpuffer vom pH 7,2) nach Abheberung des Plasmas. Die nach 5 min verfestigte Leukozytenschicht wurde als Scheibe von tiefer gelegenen Erythrozyten abgehoben und in frischer Glutaraldehydlösung weitere 30 min fixiert, 2mal in 0,2 m Saccharoselösung (pH 7,2) gespült und in dieser Lösung bei 4 °C mindestens 18 h gelagert. Kleine Segmente aus der Leukozytenscheibe wurden 2 h in 2%iger, auf pH 7,1 gepufferter OsO_4-Lösung nachfixiert und über die Acetonreihe in Epon 812 oder Araldit eingebettet. Mit Glasmessern hergestellte Semidünnschnitte wurden mit Azur II [11], mit Diamantmessern gewonnene Ultradünnschnitte mit Bleicitrat [13] und/oder Uranylacetat gefärbt.

Ferner [illegible] *Zentrifugierung* wurde Knochenmark aufgearbeitet. Bei [illegible] Untersuchungen [21] [illegible].

Zur [illegible] der [illegible] und [illegible] [illegible] und nach [illegible] werden.

Ergebnisse

[illegible]

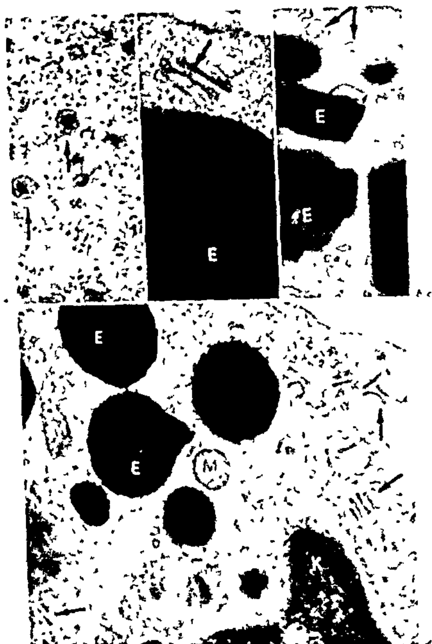
gierten Blutpräparation. Ihre Identifizierung und Abgrenzung insbesondere von Blutbasophilen war durch den Vergleich mit den orientierenden lichtmikroskopischen Befunden an Ausstrichen und Semidünnschnitten stets zweifelsfrei möglich. Struktur und Grösse der eosinophilen Granula (EG) wiesen bei Mensch und Tier die bekannten artabhängigen Differenzen auf. Soweit besondere, bisher unbekannte Formen von EG gefunden worden sind, soll deren Beschreibung gesondert erfolgen.

Der Nachweis *spezifischer Mikrogranula* (SM) gelang in den reifen Bluteosinophilen aller untersuchten Spezies. In jeder Blutpräparation wurden mindestens 25 Eosinophile mikroskopiert, welche sämtlich SM enthielten. Lediglich bei den Tigern konnten nur 5 Eosinophile pro Tier aufgefunden werden (Stresseosinopenie nach längerer Erregung und Gegenwehr beim Fang der Tiere mit Netzen), die aber ebenfalls alle SM enthielten. In ihrer einfachsten Form bilden SM runde Kugeln in einer Grössenordnung von 100 nm (Abb. 1a, 3). Ihr Inhalt ist verglichen mit dem umgebenden Grundzytoplasma relativ elektronendicht, er wird von einer Einheitsmembran umgeben, die bei höherer Auflösung eine typische Dreischichtung zeigt. Dieser einfachste Typ ist prototypisch bei der Wanderratte entwickelt und kommt auch bei den meisten übrigen Muriden vor (Tab. 1, Nr. 6–12). Nicht selten finden sich neben einfachen auch Doppelkugeln.

Von diesem Typ der Doppelsphäre leitet sich durch Längsdehnung ein weiteres, hantelförmiges Schnittprofil ab, wie es typisch bei Hund (Abb. 1d), Pferd (Abb. 1b) und etwas plumper gestaltet auch beim Menschen (Abb. 2) und einigen anderen Arten ausgeprägt ist (Tab. 1). Abgesehen von ihrer besonderen Form unterscheiden sich diese flacheren Mikrogranula durch ihren dichteren Inhalt von den Resten des bei reifen Eosinophilen weitgehend zurückgebildeten endoplasmatischen Retikulum, dessen Zisternen – am besten sichtbar am Beispiel der perinukleären Zisterne (Abb. 1d, 2) – transparent erscheinen. Hantelförmige SM stellen gestreckte Profile dar, die z. B. beim Pferd in der Länge 220 nm, in der schmalen Mittelzone 20 nm messen. Da bei den SM dieses Types offen

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Abb. 1. Spezifische Mikrogranula (Pfeile) in eosinophilen Granulozyten (E = eosinophile Granula, M = Mitochondrien, K = Kern mit z.T. tangential getroffener perinuklearer Zisterne) verschiedener Tierarten. Kugeltyp bei der Ratte *(a)*, Hanteltyp beim Pferd *(b)*, Napftyp mit U- und O-Profilen bei der Katze *(c)* und Übergänge zwischen Hantel- und Napftyp beim Hund *(d)*. Man beachte die unterschiedlichen Endvergrösserungen: bei *a–c* etwa 60000fach, bei *d* etwa 30000fach


E
E
E
E
E
E
M
K

gierten Blutpräparation Ihre Identifizierung und Abgrenzung insbesondere von Blutbasophilen war durch den Vergleich mit den orientierenden lichtmikroskopischen Befunden an Ausstrichen und Semidünnschnitten stets zweifelsfrei möglich Struktur und Grösse der eosinophilen Granula (EG) wiesen bei Mensch und Tier die bekannten artabhängigen Differenzen auf Soweit besondere, bisher unbekannte Formen von EG gefunden worden sind, soll deren Beschreibung gesondert erfolgen

Der Nachweis *spezifischer Mikrogranula* (SM) gelang in den reifen Bluteosinophilen aller untersuchten Spezies In jeder Blutpräparation wurden mindestens 25 Eosinophile mikroskopiert, welche sämtlich SM enthielten Lediglich bei den Tigern konnten nur 5 Eosinophile pro Tier aufgefunden werden (Stresseosinopenie nach längerer Erregung und Gegenwehr beim Fang der Tiere mit Netzen), die aber ebenfalls alle SM enthielten *In ihrer einfachsten Form bilden SM runde Kugeln in einer Grossenordnung* von 100 nm (Abb 1a, 3) Ihr Inhalt ist verglichen mit dem umgebenden Grundzytoplasma relativ elektronendicht; er wird von einer Einheitsmembran umgeben, die bei höherer Auflösung eine typische Dreischichtung zeigt Dieser einfachste Typ ist prototypisch bei der Wanderratte entwickelt und kommt auch bei den meisten übrigen Muriden vor (Tab 1, Nr 6–12) Nicht selten finden sich neben einfachen auch Doppelkugeln

Von diesem Typ der Doppelsphäre leitet sich durch Längsdehnung ein weiteres, hantelförmiges Schnittprofil ab, wie es typisch bei Hund (Abb 1d) Pferd (Abb 1b) und etwas plumper gestaltet auch beim Menschen (Abb 2) und einigen anderen Arten ausgeprägt ist (Tab 1) Abgesehen von ihrer besonderen Form unterscheiden sich diese flacheren Mikrogranula durch ihren dichteren Inhalt von den Resten des bei reifen Eosinophilen weitgehend zurückgebildeten endoplasmatischen Retikulum, dessen Zisternen – am besten sichtbar am Beispiel der perinukleären Zisterne (Abb 1d, 2) – transparent erscheinen Hantelförmige SM stellen gestreckte Profile dar, die z B beim Pferd in der Länge 220 nm, in der schmalen Mittelzone 20 nm messen Da bei den SM dieses Types offen-

Abb 1 Spezifische Mikrogranula (Pfeile) in eosinophilen Granulozyten (E = eosinophile Granula M = Mitochondrien K = Kern mit z T tangential getroffener perinukleärer *Zisterne*) verschiedener Tierarten Kugeltyp bei der Ratte *(a)* Hanteltyp beim Pferd *(b)* Napftyp mit U- und O-Profilen bei der Katze *(c)* und Übergänge zwischen Hantel- und Napftyp beim Hund *(d)* Man beachte die unterschiedlichen Endvergrösserungen bei *a–c* etwa 60 000fach, bei *d* etwa 30 000fach

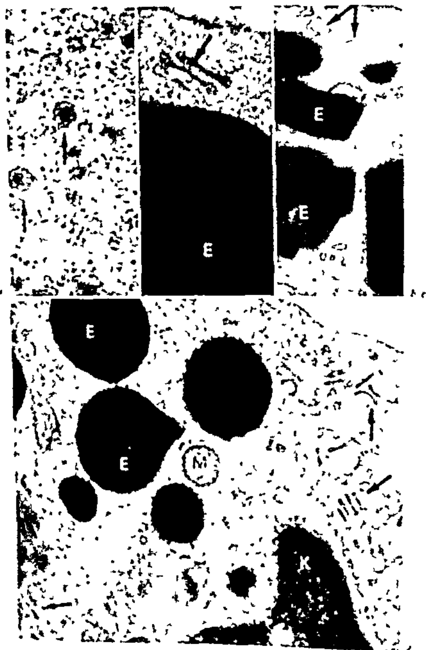

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Tabelle 1. Form und Grösse spezifischer Mikrogranula in Eosinophilen beim Menschen und einigen Säugetieren

Ordo und Spezies		Formtyp	Mittlere Grösse nm
I	Marsupialia		
	1 *Marmosa murina*	Napf (Hantel)	80/160
II	Insectivora		
	2 *Erinaceus europaeus*	Hantel	70/150
	3 *Hemiechinus auritus*	Hantel	140/290
III	Rodentia		
	4 *Sciurus vulgaris*	Hantel	20/220
	5 *Ondatra zibethica*	Hantel	15/200
	6 *Arvicola terrestris*	Kugel	70
	7 *Micromys minutus*	Kugel	45
	8 *Rattus norvegicus*	Kugel	90
	9 *Praomys morio*	Kugel	70
	10 *Praomys tullbergi*	Kugel	100
	11 *Acomys cahirinus*	Kugel	60
	12 *Acomys minous*	Kugel	60
	13 *Cavia porcellus*	Hantel	20/160
	14 *Myocastor coypus*	Hantel	30/190
IV	Carnivora		
	15 *Canis familiaris*	Hantel (Napf)	20/210
	16 *Felis catus*	Napf	20/200
	17 *Panthera tigris*	Hantel (Napf)	20/250
V	Lagomorpha		
	18 *Oryctolagus cuniculus*	Hantel	25/180
VI	Perissodactyla		
	19 *Equus caballus*	Hantel	20/220
VII	Primates		
	20 *Homo sapiens*	Hantel	20/210

Abb. 2. Spezifische Mikrogranula (Pfeile) in einem reifen Eosinophilen des Menschen (E = eosinophile Granula, K = Kern in Bildmitte tangential angeschnitten mit heller perinukleärer Zisterne, M = Mitochondrien). Endvergrösserung etwa 42 000fach.


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bar verschiedene Anschnittwinkel relativ konstant und ohne wesentliche Formabweichungen ein Hantelprofil produzieren, dürfte die räumliche Gestalt einer zentral bikonkav eingedellten Scheibe entsprechen, ähnlich etwa der Form eines Erythrozyten Mit roten Blutkörperchen haben solche SM auch eine Tendenz zu geldrollenartiger Stapelung gemeinsam (Abb 1b) Dieses Verhalten kann durch eine gewisse Adhäsionswirkung der Scheibenflächen erklärt werden

Der Hantel- oder Scheibentyp leitet durch tassenförmige Einstülpung der Scheibe zu einer dritten, napfförmigen Variante über, die prototypisch bei der Hauskatze vorkommt Je nach dem Anschnittwinkel der napfförmigen SM resultieren gewölbte U- oder ringförmige O-Profile (Abb 1c)

Bei manchen Arten kommen besonders die Scheiben- und Napfformen nebeneinander vor (Abb 1d) Bemerkenswert ist jedoch eine grundsätzliche Artabhängigkeit und Artkonstanz der verschiedenen Formtypen und des Spielraumes ihrer Dimension In ihren Extremen (z B SM von Ratte, Pferd und Katze) unterscheiden sie sich daher augenfällig (Abb 1a–c)

Von EG unterscheiden sich SM schon durch ihre mindestens um den Faktor 10 geringere Grösse, wobei Übergangsformen fehlen Von Sekundärgranula in Neutrophilen lassen sich SM durch ihre Form abgrenzen, sie haben mit diesen zwar eine vergleichbare Dimensionierung gemeinsam, eine Isomorphie besteht aber nicht oder ist allenfalls bei einigen Arten angedeutet

In eosinophilen Vorstufen des Knochenmarks fehlen SM stets Auf die Gesamtzahl aller Markeosinophilen bezogen schwankt die Häufigkeit ausgereifter und SM-haltiger Eosinophiler zwischen 5 und 10% (Anzahl mikroskopierter Eosinophiler pro Präparation mindestens 50) Bemerkenswert ist auch das häufige Fehlen von SM in scheinbar reifen Markeosinophilen, während sie in Eosinophilen aus dem Blut oder in Gewebseosinophilen (Rattenuterus Anzahl mikroskopierter Eosinophiler pro Uterus mindestens 50) regelmässig anzutreffen sind Das gilt auch für reife Bluteosinophile von Fällen chronischer Myelose oder akuter Myelose mit Begleiteosinophilie beim Menschen Offenbar bilden SM ein terminales

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Abb 3 Wandernder Eosinophiler im östrogenstimulierten Rattenuterus Pseudopodienartige Zytoplasmafortsätze enthalten nur spezifische Mikrogranula (Pfeile) eosinophile Granula (E) sammeln sich in Nähe des Kernes (K) an M = Mitochondrien, z T regressiv verändert Endvergrösserung etwa 18 000fach



bar verschiedene Anschnittwinkel relativ konstant und ohne wesentliche Formabweichungen ein Hantelprofil produzieren, dürfte die räumliche Gestalt einer zentral bikonkav eingedellten Scheibe entsprechen, ähnlich etwa der Form eines Erythrozyten. Mit roten Blutkörperchen haben solche SM auch eine Tendenz zu geldrollenartiger Stapelung gemeinsam (Abb. 1b). Dieses Verhalten kann durch eine gewisse Adhäsionswirkung der Scheibenflächen erklärt werden.

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In eosinophilen Vorstufen des Knochenmarks fehlen SM stets. Auf die Gesamtzahl aller Markeosinophilen bezogen schwankt die Häufigkeit ausgereifter und SM-haltiger Eosinophiler zwischen 5 und 10% (Anzahl mikroskopierter Eosinophiler pro Präparation mindestens 50). Bemerkenswert ist auch das häufige Fehlen von SM in scheinbar reifen Markeosinophilen, während sie in Eosinophilen aus dem Blut oder in Gewebseosinophilen (Rattenuterus: Anzahl mikroskopierter Eosinophiler pro Uterus mindestens 50) regelmässig anzutreffen sind. Das gilt auch für reife Bluteosinophile von Fällen chronischer Myelose oder akuter Myelose mit Begleiteosinophilie beim Menschen. Offenbar bilden SM ein terminales

Abb. 3. Wandernder Eosinophiler im östrogenstimulierten Rattenuterus. Pseudopodienartige Zytoplasmafortsätze enthalten nur spezifische Mikrogranula (Pfeile), eosinophile Granula (E) sammeln sich in Nähe des Kernes (K) an. M = Mitochondrien, z. T. regressiv verändert. Endvergrösserung etwa 18 000fach


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bar verschiedene Anschnittwinkel relativ konstant und ohne wesentliche Formabweichungen ein Hantelprofil produzieren dürfte die räumliche Gestalt einer zentral bikonkav eingedellten Scheibe entsprechen, ähnlich etwa der Form eines Erythrozyten Mit roten Blutkörperchen haben solche SM auch eine Tendenz zu geldrollenartiger Stapelung gemeinsam (Abb 1b) Dieses Verhalten kann durch eine gewisse Adhäsionswirkung der Scheibenflächen erklärt werden

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Differenzierungsprodukt aus der Phase des Überganges vom stabkernigen zum segmentkernigen Granulozyten Gelegentlich scheinen sich Vesikeln des in Rückbildung begriffenen Golgiapparates in SM umzuwandeln, die genaue Entstehung bedarf aber noch weiterer Klärung

Aus der Blutbahn in das Gewebe ausgewanderte und dort lokomotorisch aktive Eosinophile, wie sie unter Östrogeneinfluss im Rattenuterus auftreten, zeigen oft eine besondere Verteilung ihrer Granulation Während sich EG in der kernnahen Zytoplasmazone versammeln bevorzugen SM periphere Zytoplasmakompartimente und sammeln sich dort gern in pseudopodienartigen Fortsätzen an (Abb 3) Diese örtliche Dissoziation von EG und SM stellt ein Charakteristikum des vermutlich funktionell aktiven Gewebseosinophilen dar Bluteosinophile sind dagegen durch eine mehr homogene Verteilung von EG und SM gekennzeichnet

Diskussion

Der Nachweis eines konstanten Vorkommens von SM in reifen Eosinophilen bei 20 Säugerarten aus 7 verschiedenen Ordnungen (Tab 1) macht wahrscheinlich, dass es sich um ein zytoplasmatisches Differenzierungsprodukt handelt dem ungeachtet der möglichen funktionellen Bedeutung eine ähnliche Wertigkeit zukommt wie den seit EHRLICH [10] bekannten EG Wegen ihrer geringen Grösse sind SM nur elektronenmikroskopisch sichtbar, so dass sie von der klassischen (lichtmikroskopischen) Hämatomorphologie nicht erfasst worden sind Aber auch elektronenmikroskopisch sind SM zumeist übersehen worden, teils aus Gründen noch mangelhafter Technik, teils aus einer vorwiegenden Fixation des Interesses auf die grösseren EG

Lediglich einige Autoren haben SM (bzw entsprechende Gebilde) in Eosinophilen von Laboratoriumstieren beiläufig erwähnt So beschreibt PALADE [16] bei Ratteneosinophilen rundliche Körper, die er als besondere Ausprägungsform des glatten endoplasmatischen Retikulum auffasst POLICARD *et al* [17] erwähnen bei der gleichen Tierart besonders in Gewebseosinophilen anzutreffende «fines vesicules de tailles variables» während später ROSS und KLEBANOFF [19] am gleichen Beobachtungsobjekt ohne weiteren Kommentar von «small round granules» sprechen Offenbar ebenfalls unseren SM entsprechende «smaller granules» führen GREY und BIESELE [11] in der Abbildungslegende eines Eosinophilen der Maus auf

Neuerdings haben WARD *et al.* [24] sogenannte Sekundärgranula in Eosinophilen der Katze beschrieben, die in Form und Grösse den SM entsprechen und übrigens auch vollkommen mit sogenannten C-Granula übereinstimmen, welche WATANABE [25] bereits 1957 in Eosinophilen der Katze abgebildet hat und die zumindest grössenordnungsmässig identisch sind mit vergleichbaren Gebilden, die ebenfalls in Katzeneosinophilen von BARGMANN und KNOOP [6] als «Mikrogranula» kurz erwähnt worden sind. Die Bezeichnung «Sekundärgranula» soll andeuten, dass diese Granula als 2. Generation analog der Bildung von Sekundärgranula in Neutrophilen auftreten.

Bekanntlich sind in der neutrophilen Granulogenese im Promyelozytenstadium gebildete Primärgranula von den vorwiegend im Myelozytenstadium gebildeten Sekundärgranula unterschieden worden [9, 26, 27], die lichtmikroskopisch den azurophilen bzw. neutrophilen Spezialgranula herkömmlicher Nomenklatur entsprechen würden [1, 5]. Die Transposition jenes Prinzipes einer in 2 Generationen gestaffelten Granulogenese von der neutrophilen auf die eosinophile Reihe hat zu kontroversen Meinungen geführt. Schon 1879 hat EHRLICH [10] die Vorstellung entwickelt, dass sich die eosinophilen Granula [illegible] aus einer [illegible] Vorstufe, der [illegible] Granula [illegible] entwickle. Während [illegible] [7] hiermit übereinstimmend heute DUNN *et al.* [9] ebenso wie [illegible] [7] bei Eosinophilen nur eine Granulaart annehmen, die sich aus Progranula entwickelt haben, sich ACKERMAN [1], MILLER und HERZOG [19], BAINTON und FARQUHAR [3, 5] um den Nachweis zweier granulogenetischer Generationen auch bei der eosinophilen Reihe bemüht, wobei aber [illegible] Granula [illegible] die lichtmikroskopisch [illegible] gesprochen worden sind.

Wenn also WARD *et al.* [24] den Begriff «Sekundärgranula» auf eine ganz andere Granulationsart anwenden, so muss dies zu einer nomenklatorischen Konfusion und Irreführung Anlass geben. SM können nur dann formell als sekundäre Granulation aufgefasst werden, wenn sichergestellt ist, dass die lichtmikroskopisch sichtbare Granulation der Eosinophilen eine echte 1. Generation darstellt. Diese Frage bedarf aber offenbar weiterer Klärung, wie auch HUDSON [12] bemerkt.

Zur Vermeidung von Missverständnissen haben wir den Begriff [illegible] *der Eosinophilen* eingeführt [22]. Der Terminus «Mikrogranula» besagt, dass es sich um eine Granulation elektronenmikroskopischer Dimension handelt, die von der bisher bekannten lichtmikroskopisch sichtbaren eosinophilen Granulation [illegible] 0,5–1,2 [illegible] Granula [illegible] [1] [illegible] Ungeachtet der Möglichkeit, dass ähnliche Gebilde auch in anderen [illegible] vorkommen mögen, und das Attribut [illegible]

fisch» ausdrücken, dass es sich um ein spezifisches zytoplasmatisches Differenzierungsprodukt reifer Eosinophiler handelt, das zumindest beim Menschen und bei Säugetieren in konstanter Homologie mit speziestypischen Varietäten vorkommt. Diese besondere Granulation stellt ein morphologisches Detail der zytoplasmatischen Organisation des Eosinophilen dar, dessen funktionelle Bedeutung noch zu klären ist. Möglicherweise handelt es sich um eine Trägerstruktur solcher Enzyme, deren Vorkommen in Eosinophilen zwar bekannt ist, deren genaue Lokalisation in Zytoplasma aber noch offen ist. So wissen wir aus biochemischen Untersuchungen von LUTZNER und BENDITT [14], dass ein erheblicher Teil der sauren Phosphatase und β-Glucuronidase nicht in den EG sondern im übrigen Zytoplasma lokalisiert ist. Zytochemisch konnten wir [23] bei Ratteneosinophilen feststellen, dass insbesondere auch die alkalische Phosphatase ausserhalb der EG gelegen ist. Ob diese und möglicherweise weitere «heimatlose» Enzyme des Eosinophilen in den SM enthalten sind, wird Gegenstand weiterer Untersuchungen zur funktionellen Bedeutung dieser Körper sein.

Zusammenfassung

An 20 verschiedenen Säugerarten aus 7 Tierordnungen wird nachgewiesen, dass reife eosinophile Granulozyten ausser den bekannten eosinophilen Granula konstant über eine weitere, bisher kaum beachtete Granulationsart verfügen, die nur elektronenmikroskopisch sichtbar ist. Die Dimension solcher *spezifischen Mikrogranula* (SM) *der Eosinophilen* bewegt sich in einem Grössenordnungsbereich von etwa 100 nm. Die Eosinophilen des Menschen und der Säugetiere enthalten SM in verschiedenen artabhängigen Formvarietäten. Dabei handelt es sich um kugelige, hantel- oder napfförmige Körper, welche aus einem mässig dichten Inhalt bestehen, der von einer Einheitsmembran umgeben ist.

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Sonderdruckbestellungen an PD Dr H J Schaffer Pathologisches Institut der Universität Joseph Stelzmann Strasse 9 D 5 Köln 41 (BRD)

Acta Haemat. 50: 105–111 (1973)

Glucose-6-Phosphate Dehydrogenase Electrophoresis in Ghanaians with AA and SS Haemoglobin

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Abstract. Electrophoresis of glucose-6-phosphate dehydrogenase was carried out on 210 male Ghanaians of various ages and ethnic origins. Half of them had the normal AA haemoglobin pattern and half had sickle cell anaemia with an SS pattern. Almost half of the subjects were of Ga ethnic origin and the percentage was higher in the SS group. The overall percentage of the A type G-6-PD was 42.4. For individuals of 15 years of age or less it was 44. For individuals of Ga origin it was 46 and for the individuals with sickle cell anaemia it was 47. For individuals over 15 years of age it was 41, for non-Ga individuals it was 41 and for individuals with normal haemoglobin pattern it was 38.

Key Words
G-6-PD in Ghana
Sickle cell disease

Whereas the European usually has the B type of glucose-6-phosphate dehydrogenase (G-6-PD), the African male may have either B, A or A− types. The A− type has much less activity than A or B but the same electrophoretic pattern as A. Population surveys [1, 2] indicate that in Africans and persons of African descent the incidence of A and A− types is approximately equal (table I). Until good evidence to the contrary is forthcoming, one may expect that the percentage of G-6-PD deficiency in the male is approximately equal to half the percentage of the sum of A and A− types.

The usual rate of G-6-PD deficiency in Ghana is usually given as 22%, a figure obtained from school children of Akim or Ga ethnic origin [3]. A slightly higher figure, 24%, was obtained from school children of Akim origin [4] and a slightly lower figure, 20%, from new born infants in Accra, presumably largely of Ga origin [5]. From soldiers representing a much broader cross section of the population of the country, a figure of 14% was obtained [6]. Secondary school students in the North of the

Table I G-6-PD electrophoresis (males), relationship between A and A types

Ethnic origin	Size of sample	Percent of each type			Reference
		A	A-	B	
Yoruba (Nigeria)	141	22	22	56	1
24% Negro (Brazil)	?	8	7	85	2
Negro (USA)	311	16	18	66	1

Table II Surveys for G-6-PD deficiency in Ghana (males)

Number	Ethnic origin	Age group	Deficiency %	Reference
96	Akim + Ga	children	22	3
63	Akim	children	25	4
100	mixed	adult	14	6
131	northern	adolescent	11	7
79	*mostly* Ga	new born	20	5
470	mixed	mixed	17	weighted average

country showed only 11% with deficiency of G-6-PD [7]. The average figure for all surveys in Ghana is 17% (table II).

In Ghana, a higher than expected incidence of G-6-PD deficiency has been reported in neonatal jaundice [5], infectious hepatitis [8, 9], in typhoid fever [10] and in sickle cell disease [11]. These findings are summarized in table III. In all of the above mentioned conditions, the high incidence of G-6-PD deficiency may be due in part to the greater likelihood of such individuals to develop jaundice and thus to come to the hospital for diagnosis [12].

The report of a higher than expected incidence of G-6-PD deficiency in sickle cell trait [11] must be regarded with suspicion because most of the AA individuals were normal volunteers while many of the AS individuals had infectious hepatitis [8]. However, in Saudi Arabia such a correlation has been proven [13].

Table III G-6-PD deficiency in different diseases in Ghana

Number	Disease	Sex	Age group	Deficiency %	Reference
200	hepatitis	male and female	mixed	40	9
36	typhoid fever	male	mixed	33	10
59	neonatal icterus	male	new-born	51	5
95	sickle cell anaemia	male	mixed	43	11
450	none	male	mixed	17	table II

The assessment of an association between sickle cell anaemia and G-6-PD deficiency is more complicated. The methaemoglobin reduction test may suggest the presence of defect when it does not exist if the blood samples are not concentrated to a haematocrit of 40% [14]. The quantitative test for enzyme activity may give normal values despite an inherited defect because of the reduced average age of the red cells [15]. For these reasons, the present study was limited to electrophoresis of G-6-PD in individuals with SS and AA haemoglobin patterns.

Materials and Methods

Blood samples were collected by vein puncture from male patients attending the sickle cell clinic and the blood was placed in acid citrate dextrose (ACD) solution and kept in a refrigerator. A control series of blood samples was taken from other male patients and volunteers. The age and ethnic origin as well as the haemoglobin pattern was recorded. It was not possible to match the two series in age or ethnic origin because most of the patients in the sickle cell clinic were young individuals of Ga origin, while most of the persons in the control series were of an older age and heterogeneous ethnic origin. In fact the ethnic origin of the SS individuals was [illegible] Ga, [illegible] Akan, 7 Ewe, 2 Northern and [illegible] miscellaneous. The ethnic origin of the AA individuals was [illegible] Ga, 30 Akan, 19 Ewe, 15 Northern and 5 miscellaneous.

The technique used for G-6-PD electrophoresis has been described in a WHO Technical Report [illegible]. However, certain modifications were made. The electrophoresis was carried out at 4 °C. The dilution of the blood samples was 1:[illegible]. For staining, [illegible] mg of [illegible] was substituted for 2 mg MTT dye, and the quantity of phenazine methosulfate was reduced from 2 to 1 mg.

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Table III. G-6-PD deficiency in different diseases in Ghana

Number	Disease	Sex	Age group	Deficiency %	Reference
24	hepatitis	male and female	mixed	43	9
36	typhoid fever	male	mixed	33	10
55	neonatal jaundice	male	new born	51	5
95	sickle cell anaemia	male	mixed	43	11
470	none	male	mixed	17	table II

The assessment of an association between sickle cell anaemia and G-6-PD deficiency is more complicated. The methaemoglobin reduction test may suggest the presence of defect when it does not exist if the blood samples are not [illegible] [14]. The quantitative test for enzyme activity may give normal values despite an inherited defect because of the reduced average age of the red cells [15]. For these reasons the present study was limited to electrophoresis of G-6-PD in individuals with SS and AA haemoglobin patterns.

Material and Methods

[illegible]

[illegible]

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Table III. G-6-PD deficiency in different diseases in Ghana

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36	typhoid fever	male	mixed	39	10
49	neonatal icterus	male	new born	51	5
93	sickle cell anaemia	male	mixed	43	11
470	none	male	mixed	17	table II

The assessment of an association between sickle cell anaemia and G-6-PD deficiency is more complicated. The methaemoglobin reduction test may suggest the presence of defect when it does not exist if the blood samples are not concentrated to a haematocrit of 40% [14]. The quantitative test for enzyme activity may give normal values despite an inherited defect because of the reduced average age of the red cells [15]. For these reasons, the present study was limited to electrophoresis of G-6-PD in individuals with SS and AA haemoglobin patterns.

Materials and Methods

Blood samples were collected by venepuncture from male patients attending the sickle cell clinic and the blood was placed in acid citrate dextrose (ACD) solution and kept in a refrigerator. A control series of blood samples was taken from other male patients and [illegible]. The age and ethnic origin as well as the haemoglobin pattern was recorded. It was not possible to match the two series in age or ethnic origin [illegible] most of the patients in the sickle cell clinic were young and [illegible] while most of the persons in the control series were of an older age and [illegible] ethnic origin. In fact the ethnic origin of the SS individuals was [illegible] Ga, [illegible] Akan, 7 Ewe, 2 Northern and [illegible] miscellaneous. The ethnic origin of the AA individuals was [illegible] Ga, [illegible] Akan, [illegible] Ewe, 36 Northern and [illegible] miscellaneous.

The technique used for G-6-PD electrophoresis has been described in a WHO Technical Report [18]. However, certain changes were made. The electrophoresis [illegible] at [illegible] °C. The [illegible] of the blood samples was 1:1. For the [illegible] 1 mg of [illegible] 2 mg MTT dye and the [illegible] of phenazine methosulphate was reduced from 2 to 1 mg.

Table IV Results of G-6-PD electrophoresis (males)

Haemoglobin pattern	Age years	Ethnic origin	A type G-6-PD	B type G 6-PD
AA	15 or less	Ga	6 5[1]	7
AA	15 or less	non Ga	14 5[1]	23
AA	over 15	Ga	7 5[1]	17
AA	over 15	non Ga	11 5[1]	18
SS	15 or less	Ga	19	15
SS	15 or less	non Ga	10	19
SS	over 15	Ga	10	12
SS	over 15	non Ga	10	10

[1] One parent was Ga and one non Ga

Table V Analysis of G-6-PD type according to age, ethnic origin and haemoglobin pattern (males)

Group analyzed	A type, %	B type, %	χ^2
15 or less	44	56	0 24
Over 15	41	59	
Ga	46	54	0 8
Non Ga	40	60	
AA	38	62	1 6
SS	47	53	

Results

A total of 210 samples of blood were subjected to electrophoresis for G-6-PD (table IV) Some 42 4% showed type A while 57 6% showed type B In table V the results are grouped according to age, ethnic origin and haemoglobin pattern, with χ^2 values for each of these classifications

The difference in the percentages of types A and B between individuals 15 years of age or less, and individuals over 15 years of age is so small that it is more likely to be due to chance than to an actual difference

The difference in the percentages of types A and B between individuals of Ga and of non-Ga ethnic origin is larger but below the level of statistical significance for the sample examined.

The difference in the percentages of types A and B between individuals with normal and SS haemoglobin patterns is greater, and χ^2 analysis indicates an 80 percent probability of a real difference.

Discussion

The finding of 42.4% of type A G-6-PD in a mixed Ghanaian population (predominantly of Ga ethnic origin) suggests an incidence of G-6-PD deficiency of 21% in this population. This is slightly lower than the figures given for Ga, Ga + Akim and Akim groups but is higher than the figure of 17% for Ghana as a whole and much higher than the figure of 11% for northern Ghana. These observations are compatible with the fact that in the northern part of Ghana the incidence of the S gene is about 10% while in the southern part of Ghana it is about 20% [7], and with the observation that, populationwise, the incidence of G-6-PD deficiency parallels the incidence of the S gene [16].

The lack of variation of G-6-PD type distribution with age is compatible with all but one study [17]. The slightly higher incidence of type A G-6-PD in the Ga as compared with the non-Ga population fits in with the fact that the incidence of G-6-PD deficiency is higher in the Ga than in the non-Ga population.

The difference in incidence of type A G-6-PD in SS individuals compared with AA individuals may be due in part to the fact that there was a higher proportion of Ga individuals in the SS group.

The fact that G-6-PD deficiency causes a greater intensity of jaundice which might lead to an increased likelihood of hospital attendance and diagnosis of sickle cell anaemia is a less plausible explanation for the higher incidence of type A G-6-PD in sickle cell anaemia.

Agreement of the present results with those most recently reported [18] and discrepancy with results previously reported by the same author in an earlier study [11] may be due in part to the fact that in the earlier study there was a similar but more pronounced bias in favour of the Ga ethnic group in the SS population. However, the error may have been due in part to the experimental technique of the previous study, because severe anaemia is common in SS disease and blood from such individuals

may show less than normal G 6 PD activity despite the presence of the B type if the specimens are not properly concentrated [14]

There remains the possibility that the higher incidence of A type G 6 PD in sickle cell anaemia is due to a protective influence of this abnormality on the course of sickle cell anaemia [11, 18]

Acknowledgements The electrophoresis technique we have used was perfected by Dr BELA RINGELHANN We are indebted to Dr F I D KONOTEY AHULU and Dr CARL REINDORF for permission to use the facilities of the sickle cell clinics

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Acta Haemat 50 112–115 (1973)

Haemoglobin C in Arabs in Kuwait

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Abstract Five cases of homozygous haemoglobin C disease are described for the first time in Arabs in Kuwait One family is Jordanian the other is Kuwaiti Haemoglobin C trait has also been found in other cases

Key Words
Arabian population
Hb C disease
Hb C in Kuwait

Haemoglobin C was described by ITANO and NEEL [8] Since then it has been reported in different races and countries Negroes in West Africa [4] in South Africa [1] in North American Negroes [11, 13], in Lumbee Indians in North America [9], in Algerians [10] in Saudi Arabians [6], in an apparently Dutch [7] in Anglo Saxons [5] and in an Italian [3]

This are the first reported cases of homozygous haemoglobin C disease and trait in Arabs in Kuwait

Methods

Routine haematological tests were done according to DACIE and LEWIS [2] such as haemoglobin concentration by the cynmethaemoglobin method haematocrit reticulocyte count and osmotic fragility Fetal haemoglobin was measured according to DACIE and LEWIS [2] modification of SINGER *et al* [12] Sickling was done with *E coli* and a known positive set up at the same time Haemoglobin electrophoresis was done on cellulose acetate strips with barbitone buffer at pH 8 6 the strips were stained with Ponceau S and washed with 5% acetic acid Haemoglobin A_2 was estimated by the discontinuous buffer system tris buffer pH 9 1 at the anode and barbitone buffer pH 8 6 at the cathode The A_2 was then eluted from the strip and measured spectroscopically Only levels above 3.5% were considered to be significant

Table I

Case No	Age years	Hb %	PCV %	Retics %	HbF %	HbA_2 %	HbC %	Target cells, %	Blood film
1	2½	10.3	25	4.0	2	2.8	95	90	folded cells + hypochromia + +
2	3½	10.5	29	5.0	2	2.5	94	50	hypochromia + +
3	1½	7.4	21	2.0	2.5	2.0	95	75	hypochromia + + +
4	3	11.0	30	1.5	2	2.4	96	40	folded cells hypochromia +
5	5	10.6	25	3.0	2.5	2.8	95	60	hypochromia +

Case Reports

Case 1. The child was admitted to the hospital with jaundice. The parents stated that she has always been jaundiced since childhood, but this has increased prior to admission. History of lower limbs pain was the only significant symptom obtained. Spleen was 3.5 cm below costal margin and liver 1 cm below costal margin. The family is from Kuwait but of negro origin. The father has been married twice and the first wife is from Kuwait while the second is from Syria (mother of case 1 and 2). The family is shown in figure 1. Haematological values are shown in table I.

Case 2. Sister of case 1. She was found on family study (fig. 1). No symptoms were present, however, the spleen was 2 cm below costal margin and liver was not enlarged. Haematological values are shown in table I.

Case 3. This patient was admitted with chest infection. She was found to be anemic and, on routine examination in the Haematology Department, haemoglobin C was found. Spleen was just palpable and liver not enlarged. Parents are cousins and are from Jordan. The grandmother has haemoglobin C trait. The family is shown in figure 1 and haematological values are shown in table I.

Cases 4 and 5. Sisters of case 3. They were found on family study. In case 4 the spleen was 1.5 cm below costal margin and the liver was just palpable. In case 5 the spleen was 2.0 cm below costal margin and the liver was not enlarged.

Haemoglobin C trait was found on routine examination of blood samples for haemoglobin S in 3 more cases.

The osmotic fragility was decreased in all the cases of homozygous Hb C.

Comments

The incidence of haemoglobin C in Kuwait is about 0.6%. This may not be the true incidence since the disease is mild, as shown by the accidental discovery of the other homozygous cases. Certainly, the incidence is less than sickle cell anaemia or thalassaemia.

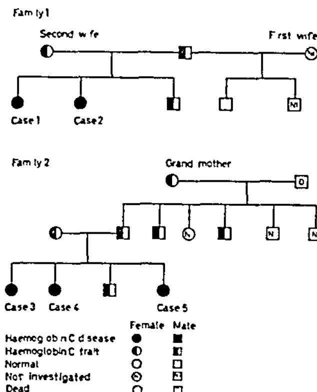


Fig 1 Pedigrees of family 1 (cases 1 and 2) and family 2 (cases 3–5)

We did not come across adult cases of homozygous haemoglobin C disease This is probably due to the low incidence of haemoglobin C, rather than the disease causing death, as it is mild and the standard of medical care in Kuwait is reasonable, so that even cases of sickle cell anaemia are living to adulthood Other workers reported adults with the disease, the oldest, reported by WHEBY *et al* [13], is 74 years old

Negroid feature was noticed in the first 2 cases and this would explain that the gene may have been imported from Africa with the old slave trade in Arabia However, the second family was Jordanian and haemoglobin C has been reported in an Italian family by DIGGS *et al* [3] The connection between the 2 nationalities is that they are from Mediterranean origin

We have not come across sickle cell haemoglobin C disease or thalassaemia haemoglobin C disease This may be due to the low incidence of haemoglobin C, it has, however, been reported by other workers

Acknowledgement We are grateful to Prof H LEHMANN for confirming the results of our electrophoresis and for his kind help

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Acta Haemat 50 116–123 (1973)

Congenital Thrombocytopathy (Platelet Factor 3 Defect) with Prolonged Bleeding Time but Normal Platelet Adhesiveness and Aggregation

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Abstract A case of platelet factor 3 deficiency (thrombocytopathy) is presented The coagulation study showed a normal plasmatic pattern but a defective prothrombin consumption The thromboplastin generation was defective too and was corrected by the substitution of the patient's platelets with normal platelets or with a platelet substitute The platelet factor 3 availability was abnormal On the contrary, platelet adhesiveness to glass and ADP, adrenaline or collagen induced platelet aggregation were normal The parents and a sister of our propositus presented a normal coagulation pattern

Key Words
Bleeding disorders
Blood coagulation
Platelet factor 3 deficiency
Thrombocytopathy

Bleeding disorders with prolonged bleeding time and no concomitant plasmatic defect still represent a very complicated matter [3, 4, 8, 14, 15, 21, 23] The recent studies seem to have confused the pattern so that any classification attempt is a very difficult task The simplest classification is the one that takes into account fundamental platelet functions and platelet number, namely clot retraction, platelet factor 3 production, platelet count Taking into account these considerations, 3 major congenital platelet syndromes may be indicated, namely thromboasthenia, thrombocytopathy and thrombocytopenia This classification is obviously approximate but it is still clinically satisfactory

Recently several mixed or 'new' syndromes have been described [13, 16–18, 20] These conditions still await final classification and may be considered, at least temporarily, as variants of one of the main conditions This appears fully justified if one takes into due account the fact that only in recent years a satisfactory platelet function tests battery has become available An exception may be postulated for the 'dystrophic thrombocy-

taire' and for the 'Portsmouth syndrome' which seem to have been satisfactorily established [1, 5, 6, 11, 25].

The object of the present paper is to present a patient with a prolonged bleeding time and an isolated platelet factor 3 deficiency, namely a case of thrombopathy or thrombocytopathy. We think, in fact, with QUICK and HUSSEY [17] and QUICK [18] that this name should be limited to conditions showing a defective platelet factor 3 activity.

Case Report

The propositus is an 11-year-old boy who was first studied by us in October 1968 and was followed ever since as an outpatient. Parents were not consanguineous and were asymptomatic. Family history was negative for bleeding disorder but for the fact that the paternal grandmother died at the age of 33 because of massive *post partum* hemorrhage.

The child was born at term and no abnormality was noted in the neonatal period. No undue bleeding was noted at the separation of the umbilical cord. At the age of 2, the patient started complaining of epistaxis. Easy bruising was first noted at approximately the same time. At the age of 7 the patient presented spontaneous bleeding from the right tonsil during an acute tonsillitis episode. The bleeding was so marked as to require admission to a local hospital where cauterization of the bleeding surface was carried out. About 2 months later, the patient presented again bleeding from the right tonsil and had to be admitted again to the local hospital. The patient was transfused with 250 ml of fresh whole blood and transferred to the Pediatric Department of our University. On the admission, the patient was not bleeding. A preliminary coagulation study showed no plasmatic abnormalities but a prolonged bleeding time and a defective prothrombin consumption. Further studies confirmed the prothrombin consumption defect and led to the diagnosis of thrombocytopathy. During admission, the patient presented 3 additional episodes of bleeding from the pharynx which promptly subsided upon transfusion of platelet-rich plasma.

During the past 3 years, the patient has been followed by us as an outpatient and no major bleeding episode occurred. Occasional epistaxis and easy bruising, however, were present.

Material and Methods

Material and methods have been discussed in detail elsewhere [9, 10]. Only new data will be given herein.

Platelet-rich plasma was obtained by centrifuging non-contacted citrated plasma at 1,000 rpm for 10 min.

Prothrombin consumption after addition of normal platelets was carried out 2 h after the standing of the blood at 37 °C. Dried thrombocytes, as supplied by Behringwerke Laboratories, Marburg, FRG, were used.

Thromboplastin generation test was carried out according to the Oxford method [2]. Patient's platelets or normal platelets were resuspended to $^1/_3$ of the original plasma volume, stirred with a glass rod for 5 min and stored at −20 °C for a few days before use. Aliquots were frozen and thawed only once.

Platelet factor 3 availability test was carried out according to the method proposed by SPAET and CINTRON [22]. Kaolin NF CB443 as supplied by Matheson Coleman & Bell, New York, was used. The Russell viper venom was used in a 1:100 000 dilution in Michaelis buffer.

Platelet adhesiveness to glass was carried out according to a modification of HELLEM's method [12]. The glass beads used were 1 mm in diameter and the height of the column was 10 cm, whereas the filtration time was 30 sec. Platelet adhesiveness was measured also according to SALZMAN's method [19]. In this latter case the material used was supplied by Becton & Dickinson Laboratories, Rutherford, N.J., USA.

Platelet aggregation was carried out according to the method of COOK and SYMONS [7], using an aggregator supplied by Evans Electroselenium Ltd., Halstead, Essex, England. The reagents supplied by Stago Laboratories, Asnières, France, were used. Adrenaline was added in the amount of 1 μg/ml of plasma or 0.5 μg/ml of plasma. Collagen was added in the amount of 40 μg/ml of plasma. The collagen solution was prepared by reconstituting the Stago reagent with 0.1 N acetic acid. The solution was then kept at 4 °C for at least 36 h. Just before use, the solution was diluted 1:10 with Michaelis buffer pH 7.3 and incubated at 33 °C for 3–7 min.

Results

The coagulation study is summarized in table I and in figure 1. The plasmatic coagulation system was normal and no hyperfibrinolysis was present. Bleeding time was prolonged whereas clot retraction was normal. Thromboplastin generation was abnormal when the patient's platelets were used in the generation mixture. The substitution of the patient's platelets with normal platelets or with a platelet substitute, corrected the abnormality (fig. 1). The Stypven clotting time was moderately prolonged and the platelet factor 3 availability test was defective. Prothrombin consumption and thromboplastin generation were defective and were corrected by the addition of normal platelets or of a platelet substitute. ADP, adrenaline and collagen-induced platelet aggregation was normal. Platelet adhesion to glass was normal with both the methods used. The administration of platelet-rich plasma corrected the prolonged bleeding time whereas the administration of platelet-poor plasma had no effect (table II).

The parents and the sister of our propositus were all found to have a normal coagulation pattern (table III).

Table I Coagulation study

Test	Propositus	Normal values	Comments
Blood platelets, $\times 10^3$/mm^3	180	150–350	normal morphology
Clot retraction	complete in 7 h	complete in 12 h	
Bleeding time (Duke), min	17–>30	3–5	extremes of several determinations
Bleeding time (Ivy), min	22	4–6	
Glass clotting time, min	7	5–9	
Prothrombin consumption, %	40	>90	
Prothrombin consumption after the addition of normal platelets, %	>90	>90	
Serum prothrombin time, sec	18.9	>22	
Serum prothrombin time after the addition of normal platelets, sec	26.6	>22	
TGT	23 sec in 6 min	<15 sec in 6 or 8 min	
TGT + normal platelets	15 sec in 6 min	<15 sec in 6 or 8 min	
TTP, sec	41.5	35–45	
Recalcification time, sec	180	100–180	
Prothrombin time, sec	13.5	13–14	
Stypven clotting time, sec	29.6	15–20	
Platelet factor 3 availability, sec	40	19	clotting times observed after 30 min incubation
Fibrinogen, mg%	400	250–450	
Factors II,V,VII,IX,X,XI, XII normal			
Thrombelastogram			
r	13	10–20	
k	6	6–12	
am	60	50–66	
Thrombin time, sec	19	18–25	
Platelet adhesiveness (Hellem mod.), %	20	10–40	
Platelet adhesiveness (Salzman), %	37	20–50	
Platelet aggregation			
ADP (5 µg)	6	6	maximal amplitude (optical density variations) in conventional units
ADP (0.5 µg)	3.7	4.7	
Adrenaline	5	6	
Collagen	6	6	

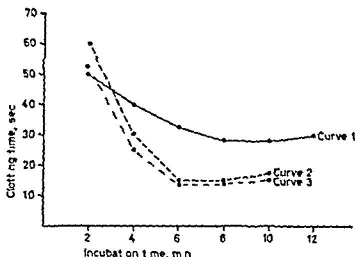


Fig 1 Thromboplastin generation test Curve 1 incubation mixture containing patient's serum, patient's adsorbed plasma and patient's platelets Curve 2 mixture containing patient's serum, patient's adsorbed plasma and normal platelets Curve 3 mixture containing patient's serum, patient's adsorbed plasma and a non activated cephalin preparation (Platelin) $CaCl_2$ was present in equal amounts in each generation mixture

Table II Bleeding time (DUKE) after plasma transfusion

	Bleeding time min	Comment
Basal value	>30, left ear	date october 25, 1968
2 h after the infusion of platelet poor plasma (260 ml)	17, right ear	no decrease in bleeding from the pharynx was noted after this transfusion
24 h after the same transfusion	22, left ear	
Basal value	>,30 right ear	date november 16 1968
2 h after the transfusion of platelet rich plasma (200 ml)	7, left ear	a decrease in bleeding was noted after this transfusion
24 h after the same transfusion	>30, right ear	

Table III. Coagulation data in the patient's relatives

Relative	Bleeding time min	Serum prothrombin time sec	Prothrombin consumption %	Platelet count ×10³ mm³	Clot retraction	PTT sec	PT sec
Father	3 5	29	>90	250	+	39	14
Mother	4	30	>90	200	+	38	13
Sister	4	28	>90	300	+	40	14
Normal values	2 5	>22	>90	150–350	+	35–45	13–14

Discussion

The main criteria for the diagnosis of platelet factor 3 defect or thrombocytopathy are normal platelet count, prolonged bleeding time, defective prothrombin consumption and normal plasmatic coagulation factors. Our patient fully meets these criteria. The possibility of an acquired condition may be ruled out because of the lifelong bleeding tendency, the lack of any associated disease and the constancy of our coagulation findings over a 4 year period. The possibility that our patient was a case of Portsmouth syndrome may be ruled out because of the normal reactivity of the platelets to adrenalin and collagen. Furthermore, in this latter syndrome the platelet factor 3 defect does not seem to be constant [6, 11, 25]. The normal platelet morphology observed in our patient, rules out the possibility of a case of '*dystrophie thrombocytaire*'. The bleeding manifestations are those seen in patients with thrombocytopathy. The prolonged bleeding time has to be ascribed only to the platelet factor 3 defect since no defect was noted in the platelet adhesiveness and aggregation or in the plasmatic coagulation pattern. This indicates that in our propositus the platelets were able to adhere normally to the wound edges but a defective 'plug' formed because of the lack of platelet factor 3. The possibility of a concomitant vascular defect, however, cannot be ruled out.

ADP release from platelets in our case was not measured but there is indirect evidence that it was normal. In fact, glass adhesion and platelet aggregation to collagen, which are phenomena known to require ADP release, were normal [24].

It was generally believed that 2 phenomena were associated [24] Our findings suggest that these 2 platelet activities may be, in certain conditions, independent In other words, a defective factor 3 production or release may be noted even though a normal ADP platelet release is present

The almost complete correction of the bleeding time after transfusion of platelet rich plasma is in agreement with our diagnosis too In this regard, it is interesting to note that 24 h after the transfusion the bleeding time was again severely prolonged This behavior is compatible with the known short survival of homologous human platelets

It has been postulated that thrombocytopathies could be divided in 2 subtypes real defect in production and defect in release We think this is irrelevant for clinical purposes Furthermore, the techniques used to free factor 3 from platelets such as sonification seem approximate at best [15] However, the results of platelet factor 3 availability test obtained in our case, strongly idicate a real deficiency rather than a defective release

As far as heredity is concerned we may only state that the normal coagulation pattern found in the propositus' father, mother and sister seems consistent with an autosomal recessive type of inheritance No other relatives of our propositus were available for study

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Acta Haemat 50 124–128 (1973)

Hereditary Persistence of Fetal Hemoglobin and β-Thalassemia in a Turkish Child

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Abstract A Turkish boy with hereditary persistence of fetal hemoglobin β thalassemia combination is described His Hb F was very much elevated and no Hb A could be shown in his hemolyzate His Hb A_2 was low normal corresponding to the Negro counterpart His father had β thalassemia trait with elevated Hb A_2 and his mother was heterozygote for high persistence fetal hemoglobin with the Hb F and Hb A_2 values resembling reported Greek AF cases

Key Words
Hemoglobin F persistence
Hemoglobinopathies
Thalassemia

Hereditary persistence of fetal hemoglobin (HPFH) was first described by EDINGTON and LEHMANN in 1955 [1] It has recently received increased attention since the heterogeneity of fetal hemoglobin in homozygotes and in β thalassemia-HPFH heterozygotes has been shown [2, 3] Most of the reported cases of HPFH [2, 3] have been in Negroes [3, 4] and Greeks [3–5] but cases among Italians [6], Sicilians [7] Porteguese-Indians [8], Puerto Ricans [9] and Thais [10] are also recorded

In homozygous HPFH cases, only Hb F is demonstrable in the red cell hemolysate, and no trace of Hb A or A_2 is present The heterozygotes have 10–38% of Hb F, which is more or less evenly distributed in the erythrocytes, with usually decreased Hb A_2 concentration Heterozygotes for HPFH β thalassemia have an average of 72% of Hb F with a normal or elevated Hb A_2 [4]

Although β thalassemia is relatively common in Turkey [11] the combination of this disease with HPFH has not been reported In fact, this is

the first report from this country of HPFH cases with or without the thalassemia combination.

Case History

H. S. (HCH 126179), a 6-year-old Turkish boy, was seen at Hacettepe Children's Hospital Medical Center because of pallor and anorexia. Paleness was first noticed at about 2 years of age and he had jaundice, for a short duration, 2 years prior being seen in this hospital.

On examination, the child looked pale and subicteric. A grade 2.6 systolic murmur was best heard over precordial area which was not transmitted. The spleen was not palpable but the liver extended 1.5 cm below the right costal margin. Other clinical findings were noncontributory.

Laboratory results: Hb 9 g/100 ml, Hct 27%, WBC 14,500/ml and a reticulocyte count 0.4%. On peripheral smear mild hypochromia, microcytosis, anisocytosis and few target cells were seen. With the exception of slight elevation of serum bilirubin (total 2.3 mg%, unconjugated 1.4 mg%), other liver function tests were within normal limits.

The patient's fetal hemoglobin (Hb F) and Hb A_2 concentrations were 82–85 and 1.75%, respectively, and no Hb A was shown by starch gel (pH 8.6) and agar gel (pH 6.45) electrophoresis. Erythrocyte osmotic fragility was markedly decreased. Inclusion bodies were not seen after staining with brilliant cresyl blue. The concentration of Hb F in red cell hemolysates of mother and father were 11.5 and 3.3%, respectively. Their Hb A_2 values were 2.42 and 5.8% in the same order. No abnormal hemoglobin could be shown in the mother's or in the father's hemolysates by starch gel and agar gel electrophoresis. However, elevated Hb F in the mother's and Hb A_2 in the father's hemolysate was readily detected by agar and starch gel electrophoresis, respectively. The mother's red cell morphology showed no abnormality and inclusion bodies with brilliant cresyl blue were not present. The osmotic fragility of red cells from both parents was slightly decreased. Staining of the peripheral smears, for fetal hemoglobin [method of KLEIHAUER and BETKE 13] revealed this hemoglobin to be uniformly distributed in all red cells of the child and his mother and only in occasional red cells of father.

Methods

Hemograms were obtained by standard techniques. The Hb concentration was determined by the cyanmethemoglobin method, and the microhematocrit was used for measuring packed cell volume. Intraerythrocytic inclusions and *fetal hemoglobin* stains were made as described by DACIE and LEWIS [12] and KLEIHAUER and BETKE [13]. Starch gel and agar gel electrophoresis were performed according to the method of SMITHIES [14] and ROBINSON *et al.* [15], respectively. In unbuffered osmotic resistance, Hb F, and Hb A_2 concentrations were determined according to DACIE and LEWIS [12], SINGER *et al.* [16] and HUISMAN and DOZY [17] successively.

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Acta Haemat. 50: 129–142 (1973)

Ultrastructural Features of Phytohemagglutinin and Concanavalin A – Responsive Lymphocytes in Chronic Lymphocytic Leukemia

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Abstract. Peripheral blood lymphocytes from patients with chronic lymphocytic leukemia (CLL) had diminished and/or delayed *in vitro* responses to both phytohemagglutinin (PHA) and concanavalin A (Con A) as determined by thymidine incorporation into DNA over a 9-day incubation period. The cytoarchitectural features of CLL lymphocytes stimulated by each of the mitogens were similar to those observed in transformed normal cells. However, as shown by planimetric measurements, the mean cell area and the nuclear, cytoplasmic and mitochondrial areas were diminished in comparison to PHA-stimulated normal cells. The data suggest that some CLL lymphocytes which transform with PHA or Con A are residual normal thymus-dependent (T) lymphocytes, and others may be derived from the metabolically defective leukemic cell population.

Key Words
Concanavalin A
Electron microscopy
Lymphocytic leukemia
Lymphocyte transformation
PHA

Incubation with phytohemagglutinin (PHA) or concanavalin A (Con A) *in vitro* induces transformation of normal human peripheral blood

[1] Recipient of a USPHS Research Career Development Award from the National Heart and Lung Institute 1 KO 4 HL 42575 04. Supported by USPHS grant AI 09334 from the National Institute of Allergy and Infectious Diseases and a research grant from the National Leukemia Association Incorporated.

[2] Supported by the Alfred und Cläre Pott Stiftung, Essen.

lymphocytes, and maximum DNA synthesis occurs at day 3 of the culture period [13]. The fundamental mechanisms underlying lymphocyte activation are yet to be determined, nevertheless, binding of the mitogen to the plasma membrane [20, 24, 27] appears to be an important requisite for the initiation of this process [15].

Lymphocytes from patients with chronic lymphocytic leukemia (CLL) have a reduced number of plasma membrane receptor sites for PHA [23] and Con A [26] and show a diminished and or delayed proliferative response to various phytomitogens *in vitro* [1, 5, 17, 21, 22, 32]. The impaired response may be the consequence of stimulation either of residual normal cells and/or of stimulation of defective leukemic cells. In order to further investigate the nature of the mitogen responsive cell population in CLL, we have incubated blood lymphocytes from normal individuals and CLL patients with PHA or Con A *in vitro* and have compared the fine structural features of transformed normal and CLL lymphocytes.

Materials and Methods

Nylon column purified peripheral blood lymphocytes from 8 healthy individuals and 13 patients with CLL (lymphocyte counts ranging from 12,400 to 49,800 /µl), were cultured for 3, 5, 7 and 9 days in the presence of PHA-P (Difco Lab., Detroit, Mich., USA) in Eagle's Minimum Essential Medium, Spinner-Modification (MEM-S, Grand Island Biol. Co., Grand Island, N. Y., USA) containing 20% fetal calf serum, 0.002 mmoles L-glutamine, 100 IU penicillin, and 100 µg streptomycin/ml. Cells from 4 of the normals and 2 of the CLL patients were stimulated with Con A (Difco, Lab., Sigma Chemical Co., St. Louis, Mo., USA). Optimal doses of the mitogens were determined by dose response curves. Details of the culture procedure have been reported [6].

Four hours before harvest, the cultures received 20 µCi ^{3}H thymidine (specific activity 2.0 Ci/mmole, New England Nuclear Corp., Boston, Mass., USA), and ^{3}H-thymidine incorporation into DNA was determined [25].

For electron microscopy, the cells were fixed in 1.5% redistilled glutaraldehyde, washed in 0.08 M sodium cacodylate, and postfixed in Millonig's phosphate buffered osmium tetroxide. The pellets were dehydrated through serial changes of ethanol followed by propylene oxide and embedded in Epon 812. Thin sections were prepared on an LKB Ultrotome III, and doubly stained with 1% uranyl acetate and lead citrate. The material was examined in a Siemens 101 electron microscope at primary magnifications from 3,000 to 40,000.

Planimetry was performed on micrographs of cells sectioned through comparable planes for resting (normal: n = 89, CLL: n = 110) and PHA activated lymphocytes (normal: n = 47, CLL: n = 55).

Table I ³H thymidine incorporation into DNA by normal and CLL lymphocytes incubated *in vitro* with PHA over 9 days (cpm/culture, values of unstimulated control cultures subtracted)

	Peripheral lymphocyte count/µl	3-day response cpm	Maximal response cpm	Day
CLL patients				
1 Sch	49 500	35	249	5
2 St	45 900	266	452	7
3 Ma	23 600	275	1 828	5
4 Ho	45 400	517	3 194	5
5 Wi	25 500	1 059	4 698	5
6 Se	14 400	3 554	8 750	5
7 We	31 300	1,140	14 864	7
8 Le	44,200	8,544	16 914	9
9 B	12,400	–	23 144	5
10 Re	46 900	5 619	28 615	5
11 Li	32 000	–	30 514	5
12 Ir	41 900	1 436	42,649	9
13 Pe	20,300	25 656	47 721	5
Normals (n = 8)				
Mean		57 749	57,749	3
SEM		8 171	8 171	

Table II ³H thymidine incorporation into DNA of normal and CLL lymphocytes incubated [illegible] Con A over 9 days (cpm/culture, values of unstimulated control cultures subtracted)

	Peripheral lymphocyte count/µl	3-day response cpm	Maximal response cpm	Day
CLL patients				
12 Ir	41 900	521	523	3
13 Pe	20 300	8,211	19 913	5
Normals (n = 4)				
Mean		67 607	67 607	3
SEM		4 105	4 105	

Table III Cell nuclear, cytoplasmic, and mitochondrial area and number of mitochondria in comparable cell sections of normal and CLL lymphocytes during a 7-day incubation with PHA

		Unstimulated		Activated by PHA					
		mean	SEM	day 3		day 5		day 7	
				mean	SEM	mean	SEM	mean	SEM
Sectional area μ^2									
Total cell	normal	24 19	4 54	134.29	10 06	117 25	4 87	64 56	4 59
	CLL	20 37	3 92	67 50	6 35	79 81	7 02		
Nucleus	normal	13 31	3 14	62 86	3 85	48 85	2 58		
	CLL	13 03	3 01	28 72	3 07	28 52	2 34	26 09	3 47
Cytoplasm	normal	10 88	3 20	71 43	7 64	68 97	4 05		
	CLL	7 33	2 72	38 78	4 86	51 29	5 19	38 47	3 19
Mitochondria	normal	1 06	0 80	7 72	0 97	7 66	1 19		
	CLL	1 37	1 10	5 32	1 04	4 51	0 68	4 53	0 94
Number of mitochondria/cell section	normal	4 92	3 49	8 55	1 12	10 92	1 06		
	CLL	5 41	4 53	10 24	1 54	9 04	1 09	14 58	3 63

Results

As shown in tables I and II, lymphocytes from the CLL patients revealed a diminished and/or delayed ^{3}H thymidine incorporation into DNA after incubation with either PHA or Con A in comparison to normal lymphocytes The magnitude and time course of the response had no relationship to the peripheral lymphocyte counts

The results of the planimetric measurements are presented in table III Unstimulated CLL cells showed only a slightly diminished mean cell area, due to a decreased cytoplasmic content After stimulation with PHA, there was an increase in the total cell area, as well as in the nuclear, cytoplasmic, and mitochondrial area and in the number of mitochondria in both normal and CLL lymphocytes The mean nuclear and cytoplasmic size, however, were considerably smaller in PHA stimulated CLL lymphocytes than in normal transformed cells, resulting in a decreased mean total cell area Although the number of mitochondria per cell profile was

similar in both transformed normal and CLL cells, their total area was diminished in CLL cells

The ultrastructural changes in normal human lymphocytes during PHA stimulation include enlargement of the nucleus with abundant euchromatin and prominent nucleoli well-developed Golgi zones, many lysosomes, and increased number and configurational changes of mitochondria, ribosomal aggregation, and some strands of rough-surfaced endoplasmic reticulum [3, 8, 9, 11, 35] In general, transformed CLL lymphocytes showed similar morphologic alterations (fig 1) however, the number of lysosomes was diminished in comparison to transformed normal lymphocytes (fig 2) In some cells, irregularly shaped nuclei, lipid vacuoles and endocytic vacuoles were prominent (fig 3) Many transformed CLL lymphocytes were smaller (intermediate-sized) than corresponding normal cells

The general cytoarchitectural features of normal lymphocytes transformed with Con A were similar to those present in PHA-stimulated cells Ribosomal aggregation was prominent in Con A-stimulated lymphocytes (fig 7) and many cells showed several strands of electron-dense endoplasmic reticulum with rare attached ribosomes (fig 5, 7, 8) Some mitochondria were vacuolated and contained areas with loss of cristae (fig 5, 7, 8) Endocytic vacuoles which contained finely granular material were present in some sections This material was also present in proximity to cell membranes and probably consisted of glycoproteins from the culture medium which were precipitated by Con A [16] (fig 5) In the Golgi region microtubules and microfilaments were discernable (fig 7) Rarely, Con A transformed normal lymphocytes showed uropod formation (fig 6) The uropods were characterized by the presence of the Golgi complex many lysosome like organelles, mitochondria and lipid bodies (fig 6) As in PHA cultures less than 20–30% of CLL lymphocytes were transformed in cultures incubated with Con A for 3 days Extensive cell agglutination was present At 3 and 5 days of incubation many intermediate sized blastoid cells were observed These cells showed only slight increase in cytoplasmic area and contained polyribosomes, few strands of rough surfaced endoplasmic reticulum, several ovoid swollen mitochondria, and a relatively small Golgi complex The large nucleus contained mainly euchromatin with some clumps of heterochromatin and prominent nucleoli Con A transformed CLL cells showed abundant ribosomal aggregation (fig 4) and in some cells formation of large endocytic vacuoles was observed (fig 4)



Fig 1 Cells from 3 day culture of CLL lymphocytes incubated with PHA The large blast like cell contains a euchromatic nucleus prominent nucleolus (N) many mitochondria (m) strands of rough surfaced endoplasmic reticulum (RER arrows) and a few granules (G) Several intermediate sized transformed cells (I) and small lymphocytes (L) are present × 5 800

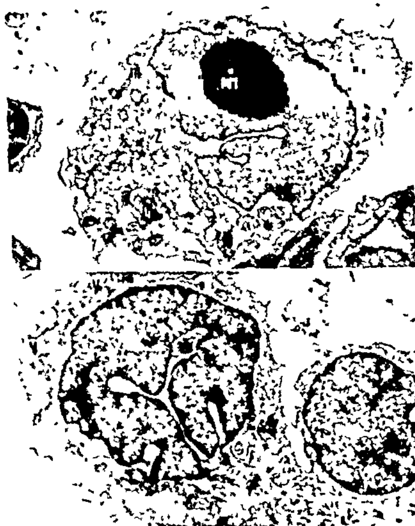
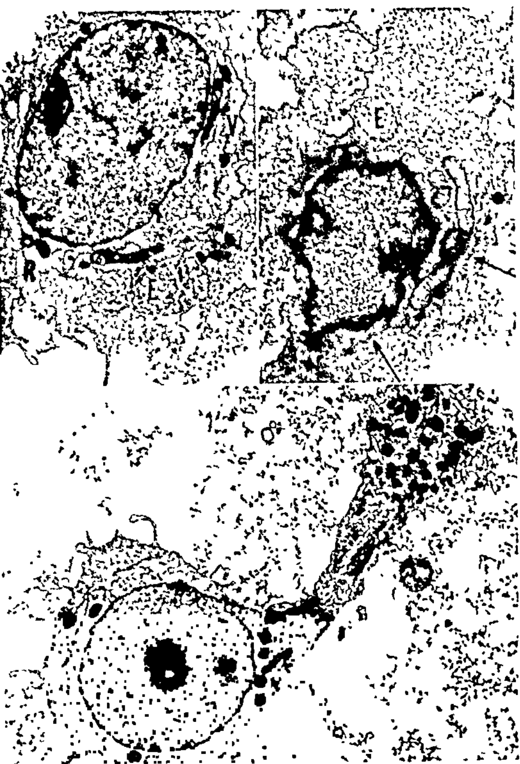


Fig. 2. Transformed cell from [illegible]-day culture of CLL lymphocytes incubated with PHA. The large nucleolus contains many nucleolonemata (N). The cytoplasm has many [illegible] (m) [illegible] of RER and a well developed Golgi zone (Z). Cytoplasmic granules are not evident in this section. × [illegible]

Fig. 3. PHA-transformed CLL cell in [illegible]-day culture. Nuclear lobulation [illegible] (V) and a large endocytic vacuole (E) are evident. × [illegible]


R
E
B

Discussion

The fine structural features of normal lymphocytes transformed with Con A have previously been briefly reported [8, 9, 12]. The sequence of morphologic events resembles that which occurs with PHA stimulation, and the spectrum of transformed cells observed is similar to that present in PHA stimulated cultures [3, 8, 9, 11, 35]. The possibility that the alterations in rough-surfaced endoplasmic reticulum and mitochondria in Con A stimulated cells are related to indirect effects of the mitogen on the cells cannot be excluded.

'Resting' blood lymphocytes from patients with CLL have been shown to differ from unstimulated normal cells in their slightly decreased mean total cell area, due to reduced cytoplasmic content [30, 31], and a diminished number of lysosomes per cell profile [10]. In the only previous electron microscopic study of PHA transformed CLL lymphocytes, CLAUSEN and BOUROWSKY [7] reported observations based on examination of 8 lymphocyte cultures obtained from one normal control and 3 patients with CLL. Although these investigators did not quantitatively assess cell or organelle size, they noted an intermediate-sized blastoid cell type and some large blastoid cells, which showed no significant morphologic differences from transformed normal cells [7]. In the present study, we have demonstrated that after stimulation with PHA *in vitro*, the area of the total cell, nucleus, and cytoplasm of many transformed CLL lymphocytes is significantly reduced in comparison to stimulated normal cells. In general, the cytoarchitectural features of the transformed cells in CLL lymphocyte cultures are similar to those of transformed normal cells except for a reduced number of lysosomes [COHNEN *et al.*, in preparation]. Furthermore, incubation of CLL lymphocytes with Con A results in transformed cells which resemble those which occur with PHA.

The majority of blood lymphocytes from most CLL patients has easily detectable surface bound immunoglobulin [for references, see 2], consid-

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Fig. 4. Con A transformed CLL cell in 5-day culture. Many ribosomal aggregates (R), lipid vacuoles (V), and endocytic vacuoles (E) are present. × 9,000.

Fig. 5. Con A transformed normal lymphocyte in 3-day culture. An endocytic vacuole (E), strands of ER with few attached ribosomes (arrows) and extracellular [illegible] material can be seen. × [illegible].

Fig. 6. Transformed cell with [illegible] from 3-day culture of normal lymphocytes incubated with Con A. × [illegible].



Fig. 7. Con A transformed normal lymphocyte in 3-day culture. In the large Golgi zone, there are microtubules (T). The ribosomes are aggregated (arrows) and many strands of endoplasmic reticulum with few attached ribosomes are present. × 6 400

Fig. 8. Cell in 5-day culture of normal lymphocytes incubated with Con A. Several mitochondria show areas devoid of cristae (arrows). × 9 600

ered to be a characteristic property of bone marrow-dependent (B) lymphocytes. Evidence from animal experiments suggests that PHA selectively activates thymus-dependent (T) lymphocytes [18]. Similarly, in mice, Con A induces proliferation of unselected T cells [19] and lymphocyte responsiveness to Con A is abolished by complete removal of T cells [33]. No significant differences were observed, however, in the average density of binding sites for Con A on T versus B lymphocytes [14, 33].

If CLL cells belong to the B cell population, then the impaired *in vitro* response to both PHA and Con A might be explained by a decreased number of normally reacting T lymphocytes. A delayed response to PHA has been reported for cultures with reduced concentrations of normal human lymphocytes [28]. The reduced reactivity of CLL lymphocytes, however, could also be related to the decreased number of membrane receptor sites for PHA [23] and Con A [26]. Since some transformed CLL cells are morphologically indistinguishable from transformed normal cells, it is likely that at least some residual normal T cells are stimulated in PHA and Con A cultures of CLL lymphocytes. Very recently [34] a mitogen responsive T cell population has been isolated from peripheral blood of CLL patients. This finding [34] is evidence that a normal population of T lymphocytes is present in CLL which is diluted by leukemic cells which have some properties of B cells [2].

Furthermore, as in cultures of PHA stimulated normal human lymphocytes [4], uropod bearing cells were observed in mitogen-stimulated CLL lymphocyte cultures (fig. 6). In guinea pigs, uropod bearing cells lack surface immunoglobulins [29], and the uropod is considered to be a characteristic of T cells. Many transformed CLL cells, however, differed from PHA or Con A transformed normal cells in nuclear and cytoplasmic size and in the number of lysosomes. These observations suggest that some transformed CLL cells probably are abnormal cells. Since various metabolic and functional abnormalities have been reported in unstimulated and stimulated lymphocytes from patients with CLL, the diminished and delayed response of CLL cells to phytomitogens appears to be at least in part the consequence of activation of defective leukemic cells. However, at present, the possibility cannot be excluded that the intermediate stimulated cells represent incompletely transformed normal cells.

Acknowledgement. We thank Miss [illegible] Mrs. [illegible] and Mrs. [illegible] for the [illegible] technical assistance.

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Acta Haemat. 50: 143-148 (1973)

Diisopropylfluorophosphate Uptake by Granulocytopoietic Cells in Chronic Myeloid Leukaemia and in Normal Individuals

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Abstract. Bone marrow cells from normal and leukaemic individuals were incubated with tritiated diisopropylfluorophosphate (DFP). Stained cells were classified cytologically and photographed. Following autoradiography the grains overlying the same cells were counted. Myeloblasts took up extremely little DFP. Promyelocytes and, to a slightly lesser extent, myelocytes incorporated DFP very actively. A lower but rather uniform activity was observed in the more mature forms. The grain count varied considerably between individual cells within each maturation compartment.

Key Words
Autoradiography
Diisopropylfluorophosphate
Granulocytopoietic cells
Myeloid leukaemia

Radioactive diisopropylfluorophosphate (DFP) has been used to label granulocytes in kinetic studies, either by intravenous infusion of DFP or *in vitro* followed by reinfusion of the labelled blood [1, 2, 4–7]. From the disappearance rates of labelled cells the intravascular granulocyte half life has been deduced.

The uptake of DFP ^{3}H by granulocyte precursors and mature cells has been investigated in an earlier study by KURTH *et al.* [8]. It was concluded that myeloblasts showed very little uptake, myelocytes a high uptake and the other cell types intermediate relative labelling. However, due to technical factors, promyelocytes and myelocytes could not be clearly distinguished.

In the present work, the DFP ^{3}H uptake was determined in cells belonging to the various granulopoietic differentiation and maturation compartments, using a two-step method which overcame this difficulty.

Materials and Methods

Three healthy volunteers were studied (No 1 2 and 3) None of them showed signs of active infection or had any history of recent infections Three Philadelphia chromosome positive patients with chronic myelogenous leukaemia (CML) in the chronic phase were selected (No 4 5 and 6) Their peripheral leucocyte counts were 6 000 12 400 and 23 900 μl respectively Differential counts of the peripheral blood in the three cases showed 9 0 and 9% of immature granulocyte precursors The haemoglobin concentrations ranged between 12.5 and 15 4 g 100 ml the platelet counts between 356 000 and 686 000 μl Patient No 6 was treated with busulphan 2 mg each day The other two patients did not receive therapy at the time of investigation

Bone marrow cells were obtained by sternal puncture The marrow was suspended in 10 ml of TC 199 medium containing 25 μCi DFP ^{3}H (The Radiochemical Centre Amersham England specific activity 3.3 Ci/mmol) and 100 IU of heparin incubated for 45 min at 37 °C and agitated intermittently to counteract sedimentation The cells were then washed twice in AB serum the cells being spun down by centrifugation at 150 g The final cell button was suspended in a volume of AB serum only slightly larger than the volume of the cells Films of this suspension were prepared on cleaned slides and quickly dried with a household hairdryer, fixed for 5 min in methanol and stained with May Grünwald Giemsa

Areas with well spread cells were photographed on Polaroid® films in a Leitz Orthoplan microscope using the 100 × oil immersion objective The cells were classified cytologically and their positions on the moving stage of the microscope were noted In each patient from 27 to 110 cells (mean 48) of each morphological type were photographed

As it was intended to correlate morphological character and DFP uptake in the cells, the next step was to prepare autoradiographs of the smears However this posed a technical problem When slides are dipped into the liquid emulsion the adhering film tends to be thicker towards the distal end of the slide The mean thickness of our films is about 2 μm as found by direct measurements and we have calculated the mean range of the β-emission from tritium to be 2.5 μm in the film using an empirical equation found by LEVI [9] which shows us that sensitivity of the emulsion varies along its length As a consequence of this we have only photographed cells in an approximately 5 mm wide band across the middle of each slide

After removal of the immersion oil in 4 changes of xylol the slides were destained in methanol containing 0.5 ml of concentrated hydrochloric acid per litre (3 changes) and finally rinsed in pure methanol After drying in air the slides were immersed in a solution containing 100 mg of gelatine and 20 mg of chromealum in 200 ml of distilled water and left to dry and harden overnight in a dust free atmosphere This greatly facilitated the adherence to the slides of the Ilford K2 liquid nuclear emulsion The emulsion was diluted 1 1 in distilled water held at 42 °C in a water bath The slides were dipped once left to gel on the table and dried at approximately 40 °C on a hotplate The drying time was about 10 min after which the slides were stored in boxes with silica gel at 4 °C and exposed for about 24 h

Table 1 The mean grain counts found in DFP labelled cells following autoradiography

Case No.	Myeloblasts mean grain count	n	range	Promyelocytes mean grain count	n	SD	Myelocytes mean grain count	n	SD	Metamyelocytes mean grain count	n	SD	Stab forms mean grain count	n	SD	Segmented mean grain count	n	SD
1	1.41	31	0–5	41.9	32	17.4	26.9	44	11.7	19.3	37	7.1	19.9	40	5.3	17.9	39	5.6
2	4.03	34	0–40	33.3	34	23.2	29.8	58	13.1	15.7	38	6.1	16.4	76	5.7	15.8	95	5.3
3	6.41	49	0–34	38.1	67	17.0	32.5	110	12.5	24.8	46	8.5	26.3	38	9.1	32.0	80	11.6
4	0.94	37	0–3	28.7	34	12.5	18.0	51	7.1	13.2	36	3.2	13.4	43	3.3	12.8	56	3.2
5	2.31	31	0–24	35.2	35	19.0	32.0	67	14.6	18.8	27	7.0	23.0	30	9.4	19.4	42	6.8
6	3.25	16	0–39	29.0	36	15.5	23.4	84	9.9	13.3	40	5.7	13.6	45	4.8	12.9	42	5.1

Cases No. 1–3 = healthy volunteers, cases No. 4–6 = patients with chronic myelogenous leukaemia, n = Number of cells investigated

Chemography or background due to mechanical stress was never observed with this procedure

The slides were developed for 15 min in a simple developer, metol 15 g and anhydrous sodium sulphite 200 g in 2 l of distilled water The slides were immersed for 2 min in a stop bath containing 2% acetic acid and fixed for 4 min in 8% sodium thiosulphate They were not agitated throughout the processing as diffusion suffices for these thin emulsions Finally the slides were rinsed twice for 10 sec in distilled water

The cells were stained through the emulsion with Leishmann's stain Those previously analyzed were easily found and the overlying grains counted The background was always less than one grain per average cell area

Cytological Classification

Myeloblasts Cells with fine leptochromatic chromatin prominent nucleoli and a light sky blue, agranular cytoplasm

Promyelocytes Usually larger than the blasts with a shallow depression in the nucleus, distinct nucleoli and only azurophilic granules in the basophilic cytoplasm

Myelocytes The chromatin is slightly more condensed Eosinophilic areas occur in the cytoplasm, which contains one or more specific granules

Metamyelocytes The nuclear chromatin is condensed nucleoli are absent and the cytoplasm eosinophilic with predominantly specific granules

Mature forms If the nucleus was more than twice longer than wide it was classified as a stabform The segmented neutrophils showed distinctly lobulated nuclei

Results

It appears from table I that the neutrophilic myeloid cells from normal individuals and from patients with CML showed essentially the same cytological distribution of DFP ^{3}H uptake

The highest mean grain count was found in promyelocytes and a somewhat lower count in myelocytes The DFP uptake in metamyelocytes and mature forms was nearly identical and on a level about half that of the promyelocytes Myeloblasts had very low grain counts

The incorporation in monocytes and eosinophils was not investigated systematically, but we have found the activity of these cells to be very low, as was the uptake by lymphocytes and erythropoietic cells, barely exceeding the background values

The standard deviation of the grain counts was considerable, particularly in promyelocytes and myelocytes In one haematologically normal individual the mean grain count was significantly higher in segmented cells than in stab- and metamyelocytes

Discussion

The present observations differ from those of KURTH *et al.* [8] in that promyelocytes showed heavier labelling than myelocytes in normal individuals as well as in CML patients. This discrepancy may be apparent rather than real. First, in the study by KURTH *et al.* [8] the difference between promyelocyte and myelocyte grain counts was only 6% of the peak value. Second, in view of the fact that these authors found it difficult to observe the cytoplasmic granules through the photographic emulsion, some promyelocytes may have been interpreted as myelocytes. Finally, the criteria for cytological classification were not defined and may have differed from those employed in the present study.

The wide scatter of the individual grain counts within the various cytological compartments cannot entirely be due to methodological factors, as cells belonging to the same cytological compartment and lying close together frequently presented widely differing grain counts.

The position of a given cell in the mitotic cycle is likely to be important in this respect as the volume of cytoplasm and the enzyme content in a premitotic G2 cell is larger than in a G1 cell, ceteris paribus; it probably takes up more DFP-^{3}H.

In one of the controls rising counts were observed in metamyelocytes, stab and segmented neutrophils. No clinical or technical factor has been found to explain this finding.

The DFP-enzyme complex is chemically very stable [2, 3]. It was consequently conceivable that the DFP-^{3}H uptake could be applied for cytological classification of metaphases prepared for karyotype analysis. However, so much of the label was lost from the cells during chromosome preparation that we were not able to carry out cytological classification of the metaphases with sufficient certainty.

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Acta Haemat. 50: 149-153 (1973)

Serum Haemopexin Concentration in Patients with Various Haemoglobinopathies

Effect of Splenectomy

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Key Words
Haemopexin
Sickle cell disease
Splenectomy
Thalassaemia

Abstract. Serum haemopexin levels were determined by the radial immunodiffusion method in 96 patients with β thalassaemia major (27 after splenectomy), in 36 with sickle cell disease and in 15 with sickle cell thalassaemia disease (4 after splenectomy). Lowering of haemopexin concentration was extremely marked in thalassaemia major and – to a lesser extent – in sickle cell and sickle cell thalassaemia disease. Comparatively higher values were noted after splenectomy.

A number of human serum proteins have the property to bind with haemoglobin or haem: haptoglobin, albumin and haemopexin. Haptoglobin binds with haemoglobin while albumin and haemopexin bind with haem to form methaemalbumin and haemopexin haem complexes, respectively. Haemopexin has been isolated and purified by SCHULTZE *et al.* [9] in 1961. The human haemopexin contains approximately 20% carbohydrates and the molecular weight is 57 000 ± 3 000 [11]. Haemopexin binds haem in a equimolar ratio [3] and the haem haemopexin complex is subsequently eliminated from the circulation and degraded mainly in the liver [8]. In healthy individuals of both sexes serum haemopexin levels range between 500 and 1 000 μg/ml [8]. Deficiency of haemopexin was noted in haemolytic anaemias including different haemoglobinopathies in a relatively limited number of patients [2, 6–8, 10].

The purpose of the present study was to extend these investigations in a larger number of patients and to compare the values between the nonsplenectomized and splenectomized patients.

Material and Methods

Patients studied included 96 cases of β thalassaemia major (27 after splenectomy), 36 cases of sickle cell disease and 18 with sickle cell thalassaemia disease (4 after splenectomy). The age of our patients varied between 2 and 27 years. A control group of 20 sera from healthy individuals aged 5–30 years was similarly investigated.

Blood was taken by venipuncture. After separation of the cells the sera were sealed and frozen at −20 °C until used. For the determination of serum haemopexin concentration the single radial immunodiffusion method was performed using commercially available immunoplates (Partigen Haemopexin, Behringwerke, Marburg, FRG). The standard curve for the determination of the haemopexin was calculated with the aid of a standardized human serum (Behringwerke). Serum immunoelectrophoresis of GRABAR and WILLIAMS [1] was carried out on microscopic slides as described by SCHEIDEGGER [12] by using specific anti-human haemopexin immunserum (Behringwerke).

Results

The mean serum haemopexin level in the 20 normal individuals was 79.20 mg/100 ml, the values ranging between 69 and 88 mg/100 ml. In the 69 cases of thalassaemia without splenectomy the mean serum haemopexin level was 13.3 mg/100 ml (range 0–28.5 mg/100 ml), while in the group after splenectomy (27 cases) it was 25.2 mg/100 ml (range 15–49 mg/100 ml). The difference between the mean haemopexin levels in normal individuals and the thalassaemia group as a whole was highly significant ($p<0.001$) as was the difference between the groups without splenectomy and after splenectomy ($p<0.001$). In the 36 cases of sickle cell disease the mean serum haemopexin level was 28.1 mg/100 ml (range 9.5–47 mg/100 ml), while in sickle cell thalassaemia the values were 13.90 mg/100 ml (range 0–27 mg/100 ml) in non-splenectomized cases and 42.5 mg/100 ml (range 40–45 mg/100 ml) in the group after splenectomy. The difference between the mean haemopexin level in normal individuals and in patients with sickle cell or sickle cell thalassaemia disease was highly significant ($p<0.001$) as was the difference between the groups without splenectomy and after splenectomy of the latter disease ($p<0.001$). No sex differences was found in any group. No attempt was made to correlate the severity of the clinical or haematological condition (especially the severity of the haemolytic process) to the values obtained.

Immunoelectrophoretic studies of all the sera investigated did not disclose any difference in the electrophoretic mobility of this protein in the

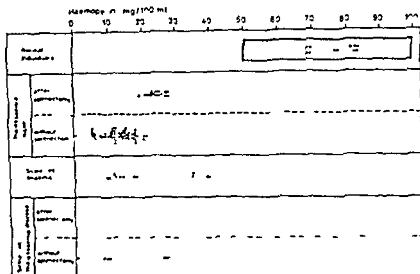


Fig. 1 Haemopexin levels in 20 normal individuals, 69 non-splenectomized and 27 splenectomized patients with thalassaemia major, 36 with sickle cell anaemia and 14 non splenectomized and 4 splenectomized patients with sickle cell thalassaemia disease

normal individuals and or the different haemoglobinopathies. The precipitation line of the haemopexin was evident only when its concentration in the serum was greater than 20 mg/100 ml. Figure 1 illustrates the results of the individual cases.

Discussion

It has long been known that haptoglobin is depleted from the plasma of patients with haemolytic diseases because of the rapid removal of the haptoglobin haemoglobin complexes. The serum of these patients may also have low haemopexin levels and haem albumin complexes usually referred to as methaemalbumin. Earlier reports demonstrated the deficiency of haemopexin in hereditary haemolytic disorders by the immunoelectrophoretic technique [6]. The advent of radial immunodiffusion technique by MANCINI *et al.* [5] incited a new interest on the behaviour of this protein in various haematologic disorders.

Material and Methods

Patients studied included 96 cases of β thalassaemia major (27 after splenectomy), 36 cases of sickle cell disease and 18 with sickle cell thalassaemia disease (4 after splenectomy). The age of our patients varied between 2 and 27 years. A control group of 20 sera from healthy individuals aged 5–30 years was similarly investigated.

Blood was taken by venipuncture. After separation of the cells the sera were sealed and frozen at −20 °C until used. For the determination of serum haemopexin concentration the single radial immunodiffusion method was performed using commercially available immunoplates (Partigen Haemopexin, Behringwerke, Marburg, FRG). The standard curve for the determination of the haemopexin was calculated with the aid of a standardized human serum (Behringwerke). Serum immunoelectrophoresis of GRABAR and WILLIAMS [1] was carried out on microscopic slides as described by SCHEIDEGGER [12] by using specific anti human haemopexin immunserum (Behringwerke).

Results

The mean serum haemopexin level in the 20 normal individuals was 79.20 mg/100 ml, the values ranging between 69 and 88 mg/100 ml. In the 69 cases of thalassaemia without splenectomy the mean serum haemopexin level was 13.3 mg/100 ml (range 0–28.5 mg/100 ml), while in the group after splenectomy (27 cases) it was 25.2 mg/100 ml (range 15–49 mg/100 ml). The difference between the mean haemopexin levels in normal individuals and the thalassaemia group as a whole was highly significant ($p<0.001$) as was the difference between the groups without splenectomy and after splenectomy ($p<0.001$). In the 36 cases of sickle cell disease the mean serum haemopexin level was 28.1 mg/100 ml (range 9.5–47 mg/100 ml), while in sickle cell thalassaemia the values were 13.90 mg/100 ml (range 0–27 mg/100 ml) in non splenectomized cases and 42.5 mg/100 ml (range 40–45 mg/100 ml) in the group after splenectomy. The difference between the mean haemopexin level in normal individuals and in patients with sickle cell or sickle cell thalassaemia disease was highly significant ($p<0.001$) as was the difference between the groups without splenectomy and after splenectomy of the latter disease ($p<0.001$). No sex differences was found in any group. No attempt was made to correlate the severity of the clinical or haematological condition (especially the severity of the haemolytic process) to the values obtained.

Immunoelectrophoretic studies of all the sera investigated did not disclose any difference in the electrophoretic mobility of this protein in the

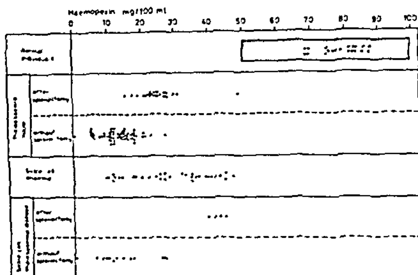


Fig. 1. Haemopexin levels in 20 normal individuals, 69 non-splenectomized and 27 splenectomized patients with thalassaemia major, 36 with sickle cell anaemia and 14 non-splenectomized and 4 splenectomized patients with sickle cell thalassaemia disease

normal individuals and or the different haemoglobinopathies. The precipitation line of the haemopexin was evident only when its concentration in the serum was greater than 20 mg/100 ml. Figure 1 illustrates the results of the individual cases.

Discussion

It has long been known that haptoglobin is depleted from the plasma of patients with haemolytic diseases because of the rapid removal of the haptoglobin haemoglobin complexes. The serum of these patients may also have low haemopexin levels and haem albumin complexes usually referred to as methaemalbumin. Earlier reports demonstrated the deficiency of haemopexin in hereditary haemolytic disorders by the immunoelectrophoretic technique [6]. The advent of radial immunodiffusion technique by MANCINI *et al.* [5] incited a new interest on the behaviour of this protein in various haematologic disorders.

The mean values of serum haemopexin in the present investigation are consistent with the findings of other investigators [2, 8]. It is also known that infants approach adult values by the end of the first year [2].

In thalassaemia major, despite the limited number of patients studied, low haemopexin levels were consistently found [2, 7, 8, 10]. Low values of this protein were associated with lowering of haptoglobin concentration [10] (although not always to the same extent) and invariably with high concentration of haem [7, 10]. The latter seems to be the most decisive factor for haemopexin depletion. In the present study the mean value for serum haemopexin in non splenectomized patients with thalassaemia major was found to be 13.3 mg/100 ml. It is probable that the higher values obtained in this study are due to the different sensitivity of the immunodiffusion method used and the greater number of patients. In splenectomized patients higher values were found and the difference was statistically significant. Sera were obtained at various times after the operation. Comparable observations of early reports are rather scanty. Muller-Eberhard and Cleve [6], by using immunoelectrophoresis, noted that deficiency of haemopexin persisted after splenectomy in 4 out of 5 patients and the same deficiency was observed in 9 additional cases studied at various time intervals after splenectomy.

In sickle cell disease low concentrations of serum haemopexin were also noted, comparable to those of thalassaemia major after splenectomy. Lower values (5–12.5 mg/100 ml) are reported by Hanstein and Muller-Eberhard in 5 patients [2], while in a more recent paper of Muller-Eberhard [8] no distinction is made between the values observed in patients with sickle cell anaemia and sickle cell haemoglobin C disease.

It was found that lowering of haemopexin level below 20 mg/100 ml was associated with complete lack of its precipitation line in the immunoelectrophoresis and it can be concluded that this level represents the limit of sensitivity of this method.

From the data of the present study and from previous reports no conclusions can be drawn as to whether the reduction of haemopexin reflects exactly the degree of haemolysis. However, it can be assumed that since lowering of haemopexin concentration is associated with high concentration of haem, measurement of the former may become a useful index of severity of the haemolytic process. On the contrary serum haptoglobin concentration is not considered as reliable index of the degree of haemolysis since this protein is easily depleted from the circulation even after minor haemolytic episodes [8]. Furthermore, from this study as well as

similar observations on the serum haemopexin level after the removal of the spleen in different haemolytic disorders [2, 6] one can assume that the increase of haemopexin level after splenectomy reflects a beneficial effect of this operation on the haemolytic process

Acknowledgment This investigation was supported by G Lyras Research grant

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Acta Haemat 50 154–161 (1973)

Splenic Function and Infection in Sickle Cell Anemia[1]

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Abstract Splenic functions have been studied in children with sickle cell anemia by determination of ^{99}Tcm uptake and release of factor VIII after adrenaline infusion A significant correlation between early cessation of splenic function and susceptibility to severe infections has been found

Key Words
Infection
Sickle cell anemia
Splenic function

Children with sickle cell anemia are very prone to infection especially with pneumococcus [5 10] In the child of pre school age, overwhelming septicemia or meningitis is a frequent cause of death When exposed to massive bacterial invasion these children are unable to cope with it and react like individuals who have had their spleens removed early in life The fulminant course of these infections suggests a defect in the immune mechanism in patients with sickle cell anemia and has been related to various abnormal splenic functions [1, 8 11, 15]

By using 2 simple, readily performed tests, we studied the phagocytic function of the spleen and measured the release of factor VIII after stress We then attempted to determine whether a relationship existed between the susceptibility to severe bacterial infections and measurable splenic functions in patients homozygous for hemoglobin S Our results indicate that patients at high risk for severe infections can be detected by splenic function tests

[1] Supported in part by USPHS grant No RR 31897 A I and USPHS research grant No P 18 HI 15170-01 from the National Institute of Health National Heart and Lung Institute Bethesda Md

Materials and Methods

We studied a total of 38 children divided into 3 groups. Group 1 consisted of 21 children homozygous for hemoglobin S as determined by hemoglobin electrophoresis. Their ages ranged from 9 months to 15 years. Group 2 was formed by 10 healthy children with a hemoglobin pattern of AA whose ages ranged between 3 and 13½ years. They served as normal controls for splenic functions. Group 3 consisted of 7 patients between 3½ and 13½ years of age who had had splenectomies in the past for various reasons. They served as controls for absent splenic functions.

All children were free of infection, and the patients with sickle cell anemia were not in crisis at the time the tests were performed. The latter were selected at random from our sickle cell clinic population. All the splenectomized patients had been followed in our hematology clinic from 1 to 5 years. After completion of the tests on all children, the hospital records of the test subjects with sickle cell anemia were reviewed to determine the number of hospitalizations for severe bacterial infections (pneumonia, meningitis). Febrile illnesses without positive X ray or bacteriological findings were not counted.

The phagocytic function of the spleen was studied by the 99m technetium ($^{99}Tc^{m}$) radioisotope uptake method. A freshly prepared solution of $^{99}Tc^{m}$ sulfur colloid was injected intravenously at a dose of 0.006 mCi/lb of body weight. After 30 min, the scintillation image (Scintiphoto) was recorded on polaroid film with a Nuclear Chicago pho gamma III scintillation camera with a thin septum multichannel low energy collimeter (Model 821742) in anterior and posterior projections. The scans were evaluated subjectively by one of us (O.S.K.) and placed in one of 3 categories: normal, decreased, or absent splenic uptake. The posterior view was found to be the most satisfactory for evaluation of the spleen. Examples of normal, decreased and absent splenic uptake of $^{99}Tc^{m}$ are given in figure 1.

The second test measured the ability of the spleen to release factor VIII after stress. LIBRE *et al.* [7] have demonstrated that the spleen, serving as a storage organ for factor VIII, releases it following adrenaline infusion in normal individuals. In contrast, splenectomized persons did not achieve significant rises of factor VIII activity in their plasma. We infused adrenaline hydrochloride in a dose of 0.0042 mg/kg of body weight at a concentration of 6.6 µg/ml in physiologic saline. The calculated dose was infused by a Harvard pump in exactly 30 min. Blood samples were collected in 3.8% sodium citrate in a relationship of 9:1 before and immediately after the infusion. The plasma was assayed for factor VIII activity by a one-stage method using partial thromboplastin time [12]. The changes in factor VIII activity after the infusion were expressed as percent rise from the pre-infusion value or if the factor VIII activity was lower following the infusion, as percent fall from the base line.

The statistical analysis of the results on the splenic release of factor VIII was carried out by the Student t test. The results are reported as the mean ± one standard deviation. For comparison of the relationship between infection and splenic function, the Fisher exact test was chosen.

predisposition was attributed to the absence of the spleen, which not only acts as a filter for bacteria from the blood stream but also plays an important role in the formation of antibodies against invading organisms. Although fatalities had occurred in older children, evaluation of 467 children after splenectomy by ERAKLIS *et al.* [4] showed clearly that the over all mortality from infection was about 2.5 times greater in children with splenectomy before the age of 4 years than if the operation was performed after that age. The report also demonstrated that mortality was much higher in children with basic underlying chronic disorders affecting the reticuloendothelial system, e.g., thalassemia, histiocytosis X and conditions associated with congestive splenomegaly, than in children who had been splenectomized for trauma to the spleen or congenital spherocytosis.

Although it was common knowledge that the spleens of patients with sickle cell anemia undergo progressive shrinkage and fibrosis, only recently has attention been focused upon the spleen in relationship to the increased susceptibility to infection in these patients. The studies of PEARSON *et al.* [8] of splenic uptake of $^{99}Tc^{m}$ showed that a large spleen in patients with sickle cell anemia was not necessarily a functioning spleen. He also demonstrated that the function could be restored temporarily in some young children with splenomegaly following transfusion of rather large amounts of normal red cells [9]. He attributed this phenomenon of functional asplenia to sluggish blood flow in the splenic cords with the biochemical environment in the spleen facilitating sickling of the red cells. Sickled cells and increased blood viscosity inherent in homozygous hemoglobin S disease led to a relative obstruction of the blood flow through the sinusoid system, thus bypassing the phagocytic cells in the spleen. Transfusion of normal cells restored the splenic circulation and the phagocytic activity temporarily. However, in children with non-palpable spleens no splenic uptake of $^{99}Tc^{m}$ could be achieved after transfusions. Whether the inability to restore the phagocytic function should be attributed to anatomic asplenia resulting from splenic fibrosis or to a total functional asplenia is difficult to evaluate.

It seems that cessation of splenic function is a gradual process, based upon the occurrence of vascular occlusion and infarction and that the time within which complete functional asplenia or splenic fibrosis develops is variable. If the process progresses slowly over a number of years, exposure to and challenge with a variety of bacterial organisms might still be met by a spleen capable of functioning at least partially, thus al-

lowing the child to build up his body defenses in an approximately normal fashion. Should, however, splenic function cease very early in life, a child with sickle cell anemia and complete functional or anatomic asplenia would face the same risks as a patient splenectomized before the age of 4 years as far as morbidity and mortality from infection are concerned. The clinical importance of detecting these endangered children is obvious. By applying both splenic function tests concurrently, we feel that we have found a valid method for detection of patients at high risk for severe infections. Although release of factor VIII by the spleen is not directly involved in primary defenses against infections, the absence of a response under stress does reveal the general impairment of the functional potential of the spleen Both tests are not only readily available and easy to perform, but can be repeated at intervals without danger to the patient. The dose of adrenaline infused does not produce undesirable side effects, such as significant rise in blood pressure and heart rate. The exposure to radiation is minimal, the amount of radiation delivered is comparable to that of an abdominal flat plate [14].

We are fully aware of the limitations of a retrospective study, but we feel that our results indicate a need for further investigation of the value of the 2 tests in detecting the cessation of splenic function in patients homozygous for hemoglobin S Patients developing functional asplenia or splenic fibrosis at an early age might benefit from monthly injections of long-acting penicillin to prevent overwhelming pneumococcal infections.

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Acta Haemat 50 162-167 (1973)

Ribonucleic Acid and Phytohemagglutinin on Rat Leukocyte Cultures within Diffusion Chambers

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Abstract RNA extracted from normal liver of inbred rats was added to *in vivo* syngeneic peripheral leukocyte cultures enclosed within diffusion chambers either 10 min after or 95 min before PHA. An increase in blastogenesis and DNA synthesis was observed, the highest values being obtained in 72 hour cultures when RNA was added after PHA. Similar results were seen with RNA pretreated with RNAse, that is, with 2S fragments. In the absence of PHA, RNA had no blastogenic effect but caused a marked increase in mononuclear phagocytic cells which could be inhibited by RNAse pretreatment

Key Words
Labeled cells
PHA
Phagocytic cells
Rat leukocyte cultures
RNA

It is well known that the addition of phytohemagglutinin (PHA) to *in vitro* or *in vivo* leukocyte cultures causes morphological [3, 6, 19, 20] and biochemical lymphocyte transformation [9, 10, 14, 15]. Many substances are capable of modifying this lymphocyte transformation and the experimental evaluation of the effect of biological agents on lymphocyte-PHA responsiveness may help to elucidate the mechanism of action. The suppression of human lymphocyte transformation by adding RNA has been reported [1, 2, 17, 18]. We have also been able to observe an inhibitory effect of high doses of RNA, although low doses of RNA proved to have a stimulating effect [7].

The object of the present paper is to investigate the effect of normal syngeneic RNA on rat leukocyte cultures, both before and after PHA stimulation. The cultures were carried out *in vivo* within diffusion chambers implanted intraperitoneally.

Methods

Animals. Male rats of the inbred G strain, weighing 200–300 g, were used.

Diffusion chambers were prepared as described by CAPALBO *et al.* [4] using Lucite rings and Millipore filters of 0.22 µm.

Heparin. 100 IU of heparin/ml of blood was added.

Culture medium. TC 199, Gibco.

Phytohemagglutinin (PHA). 0.001 ml of PHA P Difco/0.1 ml of culture medium was used.

RNA was extracted at 25 °C by the method of TRAVNICEK *et al.* [21]. 50 µg RNA/0.1 ml of culture medium was used.

Labeled DNA precursors: methyl thymidine ^{3}H (^{3}H TdR, New England Nuclear Corp., Boston). Specific activity: 6.7 Ci/mM.

Radioautographs as described by KOPRIWA and LEBLOND [11] using Kodak NTB2 emulsion.

Leukocyte cultures. For each of 3 experiments, 50 rats under ether anesthesia were bled by cardiac puncture. After sedimentation, the supernatant plasma was removed and centrifuged at 1 000 rpm for 15 min. The cell bottom was resuspended with TC 199 to a final concentration of 10^6 leukocytes/0.1 ml. The cultures were distributed into 42 Millipore diffusion chambers. No antibiotics were added. Six diffusion chambers corresponding to each group were introduced into the peritoneal cavity of 2 syngeneic male rats. Three chambers from each group (within 1 rat) were removed after 72 h and 3 after 96 h. They were treated with pronase – in order to dissolve the clot –, opened up, their content pooled (for each group), centrifuged and washed once with saline solution. The cell bottom was resuspended in 1 ml TC 199 and pulsed with 1 µCi/ml ^{3}H TdR, for 1 h.

One thousand cells were counted in the radioautographies of each sample. Whenever mononuclear phagocytic cells (MPC) were observed, they were counted separately from the lymphoid cells. The percentage of blast transformation, MPC with and without phagocytosis, labeled cells and mitotic index were determined.

The whole experiment was repeated 3 times.

The χ^2 test was used for statistical analysis.

Results

The 3 different experiments gave very similar results. These have been averaged and are summarized in table I, being subdivided into 72- and 96-hour cultures.

Discussion

Attempts to culture rat peripheral leukocytes have not always led to success [6, 19]. However, these cells have been reported to grow once

Table 1 The addition of RNA 10 min after or 95 min before rat leukocyte cultures were stimulated with PHA. Results of 72- and 96-hour cultures *in vivo* within diffusion chambers (in %)

Leukocyte cultures with	72 hour cultures						96-hour cultures					
	B	LC	Ly	MPC	LC	MI	B	LC	Ly	MPC	LC	MI
1 PHA	55	21	43	2	0	2	59	15	39	2	0	1.5
2 PHA+RNA	86	43	14	0	0	1.9	58	5	42	0	0	1
3 PHA+RNA +RNAse	82	44	18	0	0	2.5	54	0.7	46	0	0	0.9
4 RNA	0	0	72	28	0	0	0	0	78	22	1.4	0
5 RNA+RNAse	0	0	98	2	0.3	0	0	0	98	2	0.2	0
6 RNA+PHA	66	24	34	0	0	2.6	69	21	30	1	0.6	0.9
7 RNA+RNAse +PHA	65	30	33	1	0	1	63	23	36.4	0.6	0	0.7

B = blast cells, LC = labeled cells, Ly = lymphocytes, MPC = mononuclear phagocytic cells, MI = mitotic index

stimulated with PHA, both *in vitro* [16], or *in vivo* [8]. In the present experiments, it has been possible to obtain high values of blast transformation, 55 and 59%, and mitotic index, 1.5 and 2, after 72 and 96 h of PHA stimulation, respectively. These results have not been reached easily and are the culmination of a long series of experiments which led to the development of this simple, reproducible, *in vivo* technique for culturing leukocytes, without the use of antibiotics.

When syngeneic liver RNA was added to rat leukocyte cultures 10 min *after* PHA, a marked increase in blastogenesis, up to 86%, was observed in 72 hour cultures, as well as an increase in DNA synthesis, evidenced by 43% of labeled cells. Previous incubation of RNA with RNAse did not modify these results. There is no doubt that RNAse pretreatment was effective since the sucrose gradient analysis revealed that the total RNA content had been degraded in 2S fragments of polyribonucleotides. Apparently, 2S fragments would be as active as polymerized RNA in increasing PHA lymphocyte stimulation. The explanation of these results is not easy. The site of RNA action is unknown, it has been reported to be present on the cell surface as well as in the cytoplasm and nucleus of these cells [12, 13]. According to KAY [9], during PHA stimulation, there

is an alteration in the proportion of the different types of RNA synthesized and an increased rate of ribosomal and possibly of transfer RNA. It could be that RNA and or its degradation products potentiate the action of PHA at the cell surface, or even that they act as raw material, facilitating an increase in RNA synthesis within the cell. This would be accompanied by an increased DNA synthesis which would explain the higher number of ^3H TdR labeled cells in the corresponding groups.

In 96-hour cultures, both RNA and RNA pretreated with RNAse groups had returned to values similar to that of PHA-stimulated controls, but DNA synthesis was much lower. It would seem as if the optimal RNA stimulation had occurred around 72 h after the cells become exhausted with the consequent drop in mitogenesis.

When RNA was added 95 min *before* PHA, again both with and without RNAse pretreatment, an increase in blastogenesis was observed, as compared to PHA controls in 72- as well as in 96-hour cultures. In this case, the peak values of 86% observed in 72 hour cultures when RNA was added *after* PHA were not reached; on the other hand in 96 hour cultures, DNA synthesis remained high, as if the cells had not become exhausted. These results indicate that the addition of RNA *before* PHA stimulation is quantitatively less effective but more lasting than RNA added *after* PHA stimulation.

In the absence of PHA, RNA had no stimulating effect, no blast transformation or mitosis being observed. It is interesting to note, however, that mononuclear phagocytic cells (MPC) reached 28% of the cell count in 72 hour cultures and remained high at 96 h. Some of these cells had phagocytozed autologous red blood cells. Almost no labeling or mitotic figures were seen. Cytochemically MPC were peroxidase negative, which helped to discard any morphologically doubtful cell, especially from those of granulocytic origin. The number of MPC decreased to normal values, down to 2%, when RNA was previously incubated with RNAse, thus indicating that only polymerized RNA is active in this case and confirming at the same time the destructive effect of the enzyme.

It can be concluded that (1) the addition of syngeneic normal RNA to *in vitro* rat peripheral leukocyte cultures potentiates the stimulating effect of PHA; this effect is quantitatively greater, although short lived, when RNA is added 10 min *after* PHA than when it is incorporated 95 min *before* PHA. (2) The same results are obtained with RNA pretreated with RNAse, indicating that 2S fragments would be responsible for this effect. (3) In the absence of PHA, RNA has no blastogenic effect but causes an

increase in MPC, which disappear upon RNAse pretreatment In this case, polymerized RNA would be required and the mechanism of action can be said to be different in the presence or absence of PHA

Acknowledgement This investigation was supported by Fundaleu (Fundación para combatir la leucemia) The valuable technical assistance of Mr J PORTALUPPI is acknowledged

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Acta Haemat 50 168–173 (1973)

Study of the Effect of Actinomycin D on the Thrombocytopoiesis of Mice, Using [75]Se-Labelled Methionine

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Abstract After a single administration of 15 μg actinomycin D subcutaneously, the platelet count of mice fell markedly on the 5th day. At this time trombopoietic activity can be demonstrated in their serum. This is manifested in the increase of the platelet count of recipient animals and in the enhancement of the incorporation of [75]Se methionine

Key Words
Actinomycin D
Protein synthesis
Selenomethionine
Thrombocytopoiesis

On the basis of previous investigations it could be presumed that in the regulation of the platelet count an important role is played by a factor or factors either of a protein nature or bound to protein [3, 6, 9, 14, 15]

In recent years inhibition of protein formation by actinomycin D (Act D) has been widely recognized. This effect is exerted via the inhibition of RNA synthesis. The mechanism and specificity of the effect are discussed by Gellert *et al* [7]

It appeared reasonable to examine how the platelet count and thrombocytopoiesis of *experimental animals change on the administration of* Act D

Methods

The experiments were carried out on mice of the CFLP strain of both sexes with an average weight of 20 g maintained on a standard diet. The platelet count was obtained by a direct phase-contrast microscopic method [5]. The method of Penington [11] was used to study the incorporation of methionine labelled with radioactive [75]Se (SeM). Measurement was performed with an automatic sample changer connected to a Packard Tri-carb spectrometer. The only differences from the

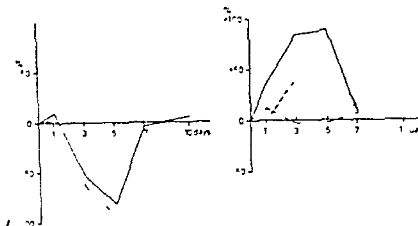


Fig. 1. Effects of various doses of Act. D on the platelet count of mice. —— = 10 μ Act. D (5 mice). —— = 15 μg Act. D (13 mice). - - = 25 μg Act. D (5 mice)

Fig. 2. Effects of serum of mice treated with 15 μg Act. D on the platelet counts normal recipients. = 48 h (5 mice), = 72 h (15 mice), —— = 5 days (15 mic and = 7 days (5 mice) after Act. D injection

original method of PENINGTON were that the material was administered intraperit neally (the results of parallel i.p. and i.v. studies were the same), while blood w not obtained from the mice by heart puncture, but from the peritoneal cavity transection of the abdominal aorta.

Results

In one case different doses of Act. D (10, 15 and 25 μg s.c.) were a ministered to three groups. The platelet counts were determined at t times given in figure 1. All of the animals to which 25 μg were admin tered died on the 5th day. The curves give the average percenta changes of the platelet counts of the mice in the individual groups. Af the administration of 15 μg Act. D marked thrombopenia was observ on the 5th day and the animals remained alive. Experiments were a carried out later with similar doses. Figure 2 shows the effects of the s um of mice treated with such doses on the platelet counts of normal rec ients. Here too the results are given as average percentage chang Transfer of serum 5 days after the administration of Act. D led to most pronounced thrombocytosis in the normal animals. Likewise on

Injection schedule			^{75}Se-meth 0.2 µCi p.	^{75}Se utilization, % × 10^{2}	n	p		n	p		n	p
15 µg Act D s.c.	■		↓	0.019 ±0.0[illegible]	8	[illegible]	0.077 ±0.01233	9		0.117 ±0.03464	9	[illegible]
Control			↓	0.057 ±0.00364	6	[illegible]	0.062 ±[illegible]	[illegible]		0.0[illegible]3 ±[illegible]	[illegible]	[illegible]
Days		−1	0	+2			+3			+5		

3

Injection schedule			^{75}Se-meth 0.2 µCi p.	^{75}Se utilization, % × 10^{2}	n	p		n	p		n	p
0.3 ml serum of Act D treated animals	■		↓	0.073 ±0.00878	9	[illegible]	0.177 ±0.0107	9	[illegible]	0.146 ±0.03015	4	[illegible]
0.3 ml normal mouse serum	■		↓	0.062 ±0.00685	3	[illegible]	0.0[illegible] ±0.0[illegible]567	3	[illegible]	0.105 ±0.0138	4	[illegible]
Days		−1	0	+2			+3			+5		

4

Injection schedule				^{75}Se-meth 0.2 µCi p.	^{75}Se utilization, % × 10^{2}	n	p		n	p		n	p
0.3 ml/day of Act D treated animals	■	■	■	↓	0.055 ±0.0110	8	[illegible]	0.100 ±0.01[illegible]6	8	[illegible]	0.173 ±0.0150	7	[illegible]
0.3 ml/day normal sera	■	■	■	↓	0.078 ±0.0[illegible]16	5	[illegible]	0.0[illegible] ±0.0[illegible]053	[illegible]	[illegible]	0.0[illegible]3 ±0.0[illegible]99	[illegible]	[illegible]
Days	−3	−2	−1	0	+1			+3			+5		

5

Fig. 3. Effect of Act. D on the ^{75}Se-methionine utilization of blood platelets of mice

Fig. 4. Effect of serum of mice treated with Act. D on the ^{75}Se utilization of the blood platelets of normal recipients

Fig. 5. ^{75}Se utilization in blood platelets of normal animals following the repeated administration of sera from Act. D treated mice

5th day after the administration of the serum (0.3 ml per mouse i.p.) when the platelet count of the donor mice was the lowest. Sera obtained 48 and 72 h after the Act. D administration also possessed activity, while the serum taken on the 7th day essentially did not change the platelet counts of the recipients.

In further experiments the direct effect of Act. D and the effect of the serum of mice treated with it on the platelet count were investigated with the incorporation of SeM. Figure 3 shows the effect of Act. D administered 48 h before the SeM. The utilization after 48 h is substantially lower, but that on the 5th day significantly higher than for the controls (which were treated only with SeM). The 5th day after the SeM corresponds to the 8th day following the administration of Act. D. At this point the platelet count of the animals already exhibits a marked increase (fig. 1).

A similar effect was also detected after the administration of the serum of mice treated with Act D The serum, which was obtained on the 5th day after administration of Act D, was given to normal mice The serum of mice treated with Act. D 48 h prior to the SeM exhibited a significant utilization increase on the 3rd day after the labelling compared to that of mice treated with normal mouse serum (fig. 4) The difference was even more considerable if the sera of normal mice and those treated with Act. D were administered repeatedly for 3 days before the SeM (fig 5)

Discussion

Numerous publications deal with the protein synthesis inhibiting effect of Act D According to the investigations of KAY [8], it also inhibits the incorporation of ^{14}C labelled leucine into the lymphocytes in spite of phytohaemagglutinin BAKKE and LAWRENCE [1] demonstrated its inhibitory effect on TSH and thyroxine formation Similar results were reported by FLEISCHER and BATTERBEE [6] in connection with ACTH formation With this method PENINGTON [12] demonstrated the thrombopoietic activity of the serum of thrombocytopenic patients His investigations indicated that the utilization was also proportional to the amount of plasma given the utilization increased after the administration of larger and repeated doses The method is generally accepted for the measurement of thrombopoietic activity [13] It is very important to choose the correct ti me interval between the times of intervention (the administration of Act D or the transfer of the serum of the animals treated with this drug) administration of SeM and the measurement of the activity in the thrombocytes, taking into consideration the time of formation and the life time of the blood platelets The 24th 48th and 72nd hours after the administration of SeM seemed the most suitable but in certain cases a significant difference can be found even on the 5th day COOPER *et al* [2] used ^{35}S-labelled sodium sulphate with which they demonstrated a thrombopoietic effect in healthy animals with the serum of thrombopenic animals (mice and rats) Studies with ^{35}S were also carried out by LAURENCE and HARKER [10] on rats subjected to exchange transfusion and hypertransfusion There was a marked activity increase in the former on the 4th day

Our studies with Act D too seem to support the view that factors of protein nature play an important role in thrombocytopoiesis and the regulation of the platelet count It is clear that with the cessation of the inhibi-

Injection schedule			⁷⁵Se meth 0 2 μCi p	⁷⁵Se utilization, % × 10⁻²	n	p		n	p		n	p
15 μg Act D s c	■		↓	0 019 ±0 00[illegible]	8	[illegible]	0 077 ±0 01133	9	[illegible]	0 117 ±0 03454	9	[illegible]
Control			↓	0 057 ±0 003[illegible]	[illegible]	[illegible]	0 062 ±[illegible]	10	[illegible]	0 063 ±0 00[illegible]	[illegible]	[illegible]
Days	-2	-1	0	+2			+3			+5		

3

Injection schedule			⁷⁵Se meth 0 2 μCi p	⁷⁵Se utilization, % × 10⁻²	n	p		n	p		n	p
0 3 ml serum of Act D treated animals	■	-	↓	0 073 ±0 00[illegible]678	9	[illegible]	0 127 ±0 0[illegible]07	9	[illegible]	0 146 ±0 03015	5	[illegible]
0 3 ml normal mouse serum	■		↓	0 062 ±0 00[illegible]5	3	[illegible]	0 0[illegible] ±0 0[illegible]67	3	[illegible]	0 105 ±0 0[illegible]	4	[illegible]
Days	-2	-1	0	+2			+3			+5		

4

Injection schedule				⁷⁵Se meth 0 2 μCi p	⁷⁵Se utilization, % × 10⁻²	n	p		n	p		n	p
0 3 ml/day of Act D treated animals	■	■	■	↓	0 055 ±0 0110	5	[illegible]	0 100 ±0 0176	8	[illegible]	0 173 ±0 0350	7	[illegible]
0 3 ml/day normal sera	■	■	■	↓	0 0[illegible] ±0 0[illegible]	5	[illegible]	0 07[illegible] ±0 00853	11	[illegible]	0 090 ±0 0069[illegible]	11	[illegible]
Days	-3	-2	-1	0	+1			+3			+5		

5

Fig 3 Effect of Act D on the ⁷⁵Se-methionine utilization of blood platelets of mice

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Our studies with Act. D too seem to support the view that factors of protein nature play an important role in thrombocytopoiesis and the regulation of the platelet count. It is clear that with the cessation of the inhibi-

tion of the protein synthesis the thrombopenia after the administration of the drug at the lowest point of which the serum of the animal possesses a marked thrombocythaemic effect may be connected with a renewed protein formation The increased uptake indicating enhanced formation and the change of the platelet count confirm this

Acknowledgement The author wishes to express his thanks to Dr L. CSERNAY Mrs I LÁSZLÓ and ÁGNES BÖRÖCZ for their valuable technical assistance

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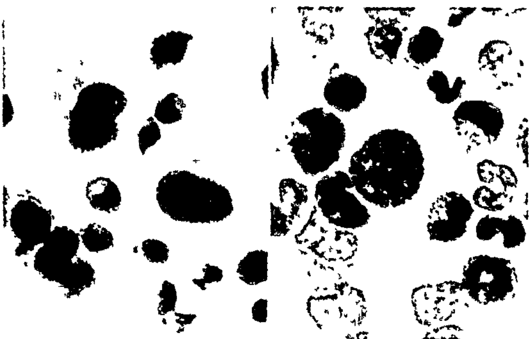


Abb 2 Unreifzellige Panmyelose Sogenannte Mikrokaryozyten im Sternalpunktat Pappenheim

mit Maximalamplitude von 6) Prothrombin 100%, Eisenspiegel 189 μg%. Im Sternalpunktat vom 25 Juli 1972 (Abb 2) ergab sich im wesentlichen keine Befundanderung Im Vordergrund standen nach wie vor mengenmassige Zunahme und Atypien der Megakaryozyten

Bei weiterhin hochgradiger und durch Prednisolon unbeeinflussbarer Thrombozytopenie wurden am 7 August 1972 erstmals Paraleukoblasten im peripheren Blutausstrich nachgewiesen (Gesamtleukozytenzahl 2400/mm³ davon 27% Paraleukoblasten) Das Sternalpunktat vom 8 August 1972 (Abb 3 4) zeigte ein sehr *polymorphes Markbild In grosser Ausdehnung sah man Verbande unreifer atypi*scher blastenartiger Zellen mit basophilem Plasma und lockerer Kernstruktur Normale granulopoetische Zellen waren stark vermindert Die Zellen der roten Reihe zeigten zum grossen Teil eine megaloblastäre Reifungshemmung mit aufgelockertem Kernchromatin und weitem Plasmasaum Megakaryozyten waren wie bei allen bisherigen Untersuchungen stark vermehrt und wiesen Zeichen der Reifungsstörung sowie Zellatypien auf

Zytochemische Untersuchungen am Sternalpunktat PAS-Farbung (Abb 5) in einem grossen Teil der Blasten deutlich granular positive Reaktion zum Teil mit mehrreihiger Anordnung der Granula In etwa 10% der Erythroblasten und megaloblastoiden Zellen stark diffus positive Plasmareaktion Gehalt der Megakaryozyten an PAS positiver Substanz wechselhaft im allgemeinen deutlich vermindert

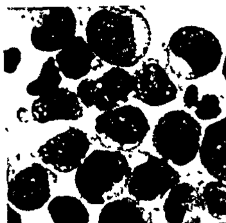


Abb. 3. Unreifzellige Panmyelose. Paraleukoblasteninfiltrate im Sternalpunktat. Pappenheim.



Abb. 4. Unreifzellige Panmyelose. Megaloblasten im Sternalpunktat. Pappenheim.

Eisennachweis: Im Markretikulum in mässiger Menge Eisenpigment nachweisbar. Kaum Sideroblasten.

Saure Phosphatase (Abb. 6): normale Aktivität in den Zellen der Restgranulopoese. Intensive diffuse Plasmareaktion in den Blasten. Umschriebene paranukleäre Fermentaktivität in einem Teil der Megaloblasten. Hohe Aktivität in den meisten Megakaryozyten.

α-Naphthylacetat-Esterase (Abb. 7): In einem Teil der Blasten mittelstarke bis starke Enzymaktivität mit diffuser Verteilung im Zytoplasma. Nur in ganz vereinzelten Erythroblasten Enzymaktivität nachweisbar. Megakaryozyten wechselhaft positiv.

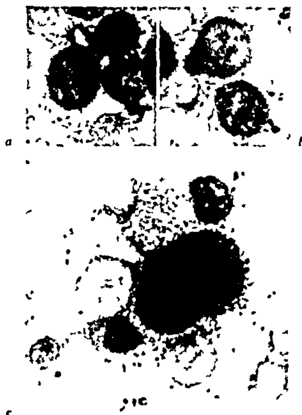


Abb. 5. Unreifzellige Panmyelose. Paraleukoblasten mit granulär positiver Reaktion (*a, b*) und atypische Megakaryozyten mit vermindertem Glykogengehalt. Sternalpunktat. PAS

Naphthol-AS-Azetat-Esterase: in einem Teil der Blasten granulär positive Reaktion, durch Natriumfluorid völlig hemmbar.

Naphthol-AS-D-Chlorazetat-Esterase (Abb. 8): neben den Zellen der Restgranulopoese lediglich Darstellung eines kleinen, offenbar promyelozytär differenzierten Anteils der Blasten.

Peroxydase: nur in wenigen Blasten Fermentaktivität nachweisbar.

β-Glukuronidase: alle Blasten negativ.

Therapie und Verlauf. Die Behandlung erfolgte mit verschiedenen Zytostatika-Kombinationen (L-Asparaginase, Vincristin, Daunoblastin, Amethopterin) in Verbindung mit Prednisolon, Antibiotika und Frischbluttransfusionen. Eine Remission war jedoch nicht zu erzielen. Der Patient verstarb bei unbeeinflussbarer Granulozytopenie und Thrombozytopenie in der 20. Behandlungswoche. Im postmortal gewonnenen Milz-

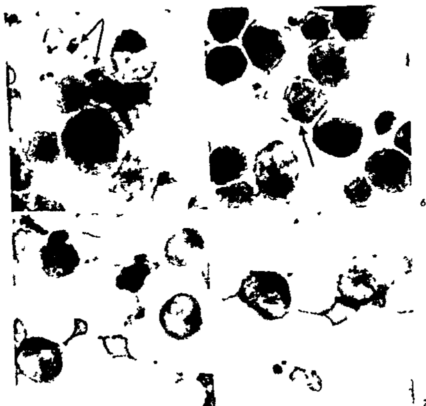


Abb. 6. Unreifzellige Panmyelose. Intensive diffuse Plasmareaktion in den Paraleukoblasten und umschriebene paranukleare Reaktion in den Megaloblasten (→). Sternalpunktat. Saure Phosphatase.

Abb. 7. Unreifzellige Panmyelose. Blasteninfiltrate mit teils hoher, teils fehlender Fermentaktivität. Sternalpunktat. α-Naphthylacetat-Esterase.

punktat (Abb. 9) beeindruckte ein buntes Gewebsbild, das die Proliferation verschiedener Zellformen erkennen liess.

Diskussion

Auffälligstes zytologisches Merkmal war bereits im ersten Sternalpunktat zum Zeitpunkt der klinischen Manifestation der Erkrankung

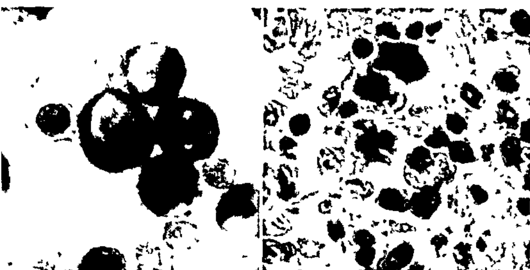


Abb. 8. Unreifzellige Panmyelose. Blasteninfiltrat mit mehreren promyelozytär differenzierten Paraleukoblasten. Sternalpunktat. Naphthol-AS-D-Chlorazetat-Esterase.

Abb. 9. Unreifzellige Panmyelose. Milzpunktat (postmortal). HE.

eine ausgeprägte Megakaryozytose mit Zellpolymorphie und -atypien. Dieser Befund war während des ganzen Krankheitsverlaufs bis zum Tode ebenso konstant nachweisbar wie die deutliche Megaloblastose. Blasten traten anfangs nur vereinzelt und verstreut auf und waren daher in ihrer biologischen Wertigkeit zunächst nicht sicher deutbar. Auch als später Paraleukoblasten ausgeschwemmt wurden und somit der periphere Blutausstrich für eine unreifzellige Leukose sprach, bestand im Knochenmark nicht das uniforme Bild einer diffusen leukämischen Infiltration. Auch bei zytomorphologisch typischen unreifzelligen Leukosen ist die maligne Entartung durchaus nicht immer auf das leukopoetische System beschränkt. Proliferationskinetische Untersuchungen [4] zeigen vielmehr, dass sich auch die roten Vorstufen an der Reifestörung beteiligen und dass demnach die Anämie nicht allein mit einer Verdrängung der Erythropoese zu erklären ist. Schon die gewöhnliche lichtmikroskopische Betrachtung des Sternalmarkausstrichs lässt diese Reifungshemmung erkennen. Wenn man von therapiebedingten Megaloblastosen absieht, wie sie beispielsweise nach Applikation des Antimetaboliten Cytosinarabinosid zu sehen sind [15], kann man recht häufig auch Erythroblasten mit spontan aufgetretener Reifungsdissoziation zwischen Kern

und Plasma [14] und mit allen Übergängen bis zu typischen Megaloblasten als Einzelexemplare sehen. Selten handelt es sich sogar um quantitativ erhebliche sekundäre Megaloblastosen mit perniziosaähnlichem Markbild [5, 12, 13]. Der für dieses Phänomen mitunter gebrauchte Begriff der «Aufbrauchperniziosa» ist schlecht gewählt, weil es sich nicht um Folgen eines gesteigerten Vitamin-B_{12}- und Folsäureverbrauchs durch die wuchernden Paraleukoblasten handelt, sondern um eine dem Leukoseprozess parallel verlaufende Ausreifungsstörung im erythropoetischen System. Das geht schon daraus hervor, dass sich die Veränderungen gegenüber einer antiperniziösen Behandlung als refraktär erweisen.

Wesentlich seltener sieht man bei unreifzelligen Leukosen auch eine Reifungshemmung der Megakaryozyten [1, 12], die ja in der Regel hochgradig vermindert sind. Diese kleinen, nur noch an der typischen Farbbarkeit und Struktur des Zytoplasma als Varianten des Megakaryozyten erkennbaren sogenannten «Mikrokaryozyten», die oft kaum grösser als Monozyten sind und meist einen unsegmentierten runden oder ovalen Kern haben, sind aber ein sehr häufiger, fast regelmässig anzutreffender Befund bei der chronischen Myelose [1].

In unserem Falle waren die pathologischen Veränderungen am erythropoetischen und megakaryozytären System qualitativ und quantitativ so ausgeprägt, dass man nicht von Begleiterscheinungen einer unreifzelligen Leukose sprechen kann, sondern eine gleichgeordnete maligne Entartung aller drei blutbildenden Zellreihen annehmen muss, zumal die Atypien der Megakaryopoese zeitlich eher fassbar waren als die der Leukopoese. Mit der von Löffler und Leder [8] publizierten Beobachtung einer Panmyelose besteht zytologisch weitgehende Übereinstimmung. Zytochemische Unterschiede betreffen vor allem die Erythroblasten. Diese zeigten bei Löffler und Leder in einem wesentlich höheren Prozentsatz Siderosomen und wiesen eine hohe Aktivität an α-Naphthylazetat-Esterase auf. In unserem Falle waren von den Befunden, die für eine maligne Entartung der Erythropoese kennzeichnend sind [2, 6, 7, 10], nur an einem Teil der Zellen die Reaktionsausfälle der sauren Phosphatase und der PAS-Färbung typisch.

Die Paraleukoblasten liessen sich nach ihrem zytochemischen Verhalten nicht in die üblichen Klassifizierungsschemata unreifzelliger Leukosen einordnen und waren auch untereinander different. Unter Hinweis auf eine ähnliche Beobachtung zytochemisch heterogener Blasten [9] mag die Frage berechtigt sein, ob diese Zellen überhaupt aus einer einheitlichen Zellpopulation hervorgegangen sein können.

Zur Terminologie wäre anzumerken, dass der Begriff «Panmyelose» [8] missverständlich ist, da er auch für die Hyperplasie aller 3 hämopoetischen Systeme im Knochenmark bei Polycythaemia vera gebraucht wurde [3]. Durch den Zusatz «unreifzellig» ist die Erkrankung zytologisch, nosologisch und zugleich prognostisch hinreichend charakterisiert.

Therapeutische Erfahrungen bestehen angesichts der extremen Seltenheit dieser Hämoblastoseform nicht. Da klinische Symptomatik, Verlauf und Prognose Unterschiede zu unreifzelligen Leukosen nicht erkennen lassen, sollten auch die dort geltenden Behandlungsgrundsätze angewandt werden. Auf zwei Gesichtspunkte, die den Therapieplan betreffen, sei abschliessend hingewiesen. Einmal sollte eine ätiologisch unklare Megakaryozytose mit Zellatypien als erstes Hinweiszeichen auf eine Hämoblastose ernst genommen und durch kurzfristig wiederholte Sternalpunktionen im Verlauf verfolgt werden, damit der Beginn der zytostatischen Therapie nicht verzögert wird. Die beschriebenen Atypien kommen nur in ganz wenigen Fällen bei nicht leukämischen Erkrankungen vor [1]. Ferner sollte eine Megaloblastoidie bei hämatologischen Systemerkrankungen nicht zur Vitamin-B_{12}-Therapie verleiten. Diese ist nicht nur wirkungslos, sondern kontraindiziert, da ja die DNS-Bildung gerade blockiert werden soll [11].

Zusammenfassung

Es wird über klinisches Bild, zytologische und zytochemische Befunde einer seltenen Form einer unreifzelligen Hämoblastose berichtet, bei der eine maligne Entartung der Leuko-, Erythro- und Megakaryopoese vorlag.

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Zur Terminologie wäre anzumerken, dass der Begriff «Panmyelose» [8] missverständlich ist, da er auch für die Hyperplasie aller 3 hämopoetischen Systeme im Knochenmark bei Polycythaemia vera gebraucht wurde [3]. Durch den Zusatz «unreifzellig» ist die Erkrankung zytologisch, nosologisch und zugleich prognostisch hinreichend charakterisiert.

Therapeutische Erfahrungen bestehen angesichts der extremen Seltenheit dieser Hämoblastoseform nicht. Da klinische Symptomatik, Verlauf und Prognose Unterschiede zu unreifzelligen Leukosen nicht erkennen lassen, sollten auch die dort geltenden Behandlungsgrundsätze angewandt werden. Auf zwei Gesichtspunkte, die den Therapieplan betreffen, sei abschliessend hingewiesen. Einmal sollte eine ätiologisch unklare Megakaryozytose mit Zellatypien als erstes Hinweiszeichen auf eine Hämoblastose ernst genommen und durch kurzfristig wiederholte Sternalpunktionen im Verlauf verfolgt werden, damit der Beginn der zytostatischen Therapie nicht verzögert wird. Die beschriebenen Atypien kommen nur in ganz wenigen Fällen bei nicht leukämischen Erkrankungen vor [1]. Ferner sollte eine Megaloblastoidie bei hämatologischen Systemerkrankungen nicht zur Vitamin B_{12}-Therapie verleiten. Diese ist nicht nur wirkungslos, sondern kontraindiziert, da ja die DNS-Bildung gerade blockiert werden soll [11].

Zusammenfassung

Es wird über klinisches Bild, zytologische und zytochemische Befunde einer seltenen Form einer unreifzelligen Hämoblastose berichtet, bei der eine maligne Entartung der Leuko-, Erythro- und Megakaryopoese vorlag.

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Table I. Hematologic findings (values in parentheses indicate our normals)

	Patient	Mother	Father	Brother
Hb g%	6.12	12.21	10.96	10.16
Hct %	19	38	35	
Reticulocytes %	2.6	–		–
Leukocytes per mm³	30,600			
Sickling test	neg.	neg.	neg	neg
Coombs test	neg	–		
Autohemolysis[1]	normal	normal	normal	normal
Erythrocyte fragility	markedly decreased	decreased	decreased	decreased
Plasma hemoglobin mg% (<5 mg%)	12			
Serum iron µg%	344	93[2]	206	
Serum iron binding capacity µg%	362	346	304	
Inclusion bodies ( )[3]	+++	+	rare	rare
Hb A_2 % (1.9–3%)	2.35	1.35	4.72	5.1
Hb F % (<2%)	14.5	2.3	3.4	3.6

[1] With and without glucose.
[2] She was 7 months pregnant.
[3] Intraerythrocytic inclusion bodies appeared after incubation with 1% brilliant cresyl blue for 30 min at 37 °C.

that at 7 months of age he was found to be severely anemic in another hospital and was given several transfusions.

On examination he appeared pale, chronically ill and underdeveloped (height 87 cm, weight 11.5 kg, both below the third percentile). The liver extended 2.5 cm and the spleen 4 cm below the costal margins. Other physical findings were unremarkable and no facial or skull deformities or jaundice were noted.

Laboratory examination revealed marked anemia and stained blood films showed hypochromia, poikilocytosis, polychromasia and a few target and nucleated red blood cells ([illegible] of 100 WBC). Additional hematologic data including that of family members are given in table I. Starch gel hemoglobin electrophoresis (pH 8.6, t[illegible]) disclosed a minor fast component in addition to Hb A and Hb A_2. On agar gel (pH 6.45, citrate phosphate) an elevated Hb F component was separated from the major fraction (fig. 1). Starch gel electrophoresis at pH 7 (phosphate buffer) indicated the presence of an anodal band corresponding to Hb H. [illegible] Serum proteins and repeated urine analyses were normal.

The parents were from a small town but denied consanguinity. Three siblings, who died at [illegible], 1.5 and 2 years of age, had manifestations similar to the patient, and a 7-year-old brother received 7 blood transfusions because of pallor (fig. [illegible]). The mother, father and the brother have a normal Hb electrophoresis,

Acta Haemat. 50: 184–190 (1973)

Hemoglobin H-β-Thalassemia

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Abstract. A Turkish child with Hb H-β-thalassemia and a clinical picture of thalassemia major, is described. His father and brother have α-β-thalassemia with hematologic findings no more severe than would be expected if they had either trait alone, and his mother has α-thalassemia alone. History revealed that 3 siblings have died from a similar illness in early infancy, perhaps indicating the severity of the combination in this family.

Key Words
Hb H-β-thalassemia
Hemoglobinopathies
Thalassemia

The thalassemias are characterized by a discordant rate of synthesis of structurally normal polypeptide chains, resulting in a deficiency of the hemoglobin, associated with the chain involved. So far α-, β- and δ-thalassemias, and their combinations, are well recognized, γ-thalassemia has been hypothesized [1] and a case of γ-β-thalassemia has been well documented [2].

Here, we are reporting a child with thalassemia syndrome, whose hemoglobin and family studies are most compatible with the diagnosis of Hb H-β-thalassemia, not previously reported to our knowledge.

Patient Report

A.I. (HCH 261,514), a 3-year-old boy from the southern part of Turkey, was admitted to Hacettepe Children's Hospital Medical Center with complaints of pallor, lassitude and puffiness of the eyes since early infancy. Past history disclosed

pattern but Hb A_2 values of the mother was definitely decreased and the father's and brother's were elevated.

Methods

Hemograms were obtained by the standard techniques. Hemoglobin (Hb) concentrations were determined by the cyanmethemoglobin method and the microhematocrit was used for measuring packed cell volume. Intraerythrocytic inclusion stain, autohemolysis (with and without glucose) and erythrocytic osmotic fragility were done as described by DACIE and LEWIS [3] and the sickling test by using 2% sodium metabisulfite. Serum iron, total iron binding capacity and plasma hemoglobin concentrations were determined according to SIMMONDS [4], PETERS *et al.* [5] and CROSBY and FURTH [6] respectively. Starch gel and agar gel electrophoresis were performed by the methods of SMITHIES [7] and ROBINSON *et al.* [8] successively. Hb F values were measured by the alkali denaturation method of SINGER *et al.* [9] and quantitative estimation of Hb A_2 was obtained by chromatography on diethylaminoethyl cellulose [10].

Comments

Thalassemia syndromes are not rare in Turkey. 137 electrophoretically-studied Cooley's anemia cases were collected at this institution between 1963 and 1971 [11]. Although α thalassemia is much less common, 4 families with Hb H disease [12] and 2 families with sickle-cell-α-thalassemia combination have been observed.

Although the pathogenesis of thalassemias has been discussed on the messenger RNA level [13, 14] there is still no single practical test to establish and or differentiate the specific type. The differential diagnosis of thalassemias remains largely dependent upon family studies. The most interesting aspect of this family is the observation of different combinations of α- and β thalassemias. The propositus had Hb H, which indicates his double heterozygosity for α-thalassemia (α-thal$_1$ + α-thal$_2$). He differs from other patients with Hb H disease in severity of anemia and elevation of Hb F. Hb F measured by the method of SINGER *et al.* [9] may include not only fetal hemoglobin, but some proportion of Bart's hemoglobin since it is also alkali resistant [15]. But elevated Hb F was also shown by agar gel electrophoresis. Since the father carries a β thalassemia gene indicated by elevated Hb A_2, the increased Hb F level of the patient is explained by the presence of the β thalassemia gene. The father must also be carrying the α-thalassemia gene since the propositus has Hb H. Rare inclusion bodies seen in the erythrocytes are additional

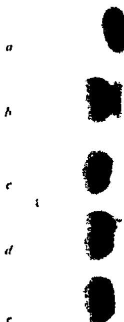


Fig 1 Agar gel (pH 6.45) hemoglobin electrophoresis (from top) *a* Thalassemia major hemolyzate only Hb F present *b* A F (the propositus) Hb A and elevated Hb F *c–e* Father mother and the brother's hemolyzates showing Hb A and a normal quantity of Hb F respectively

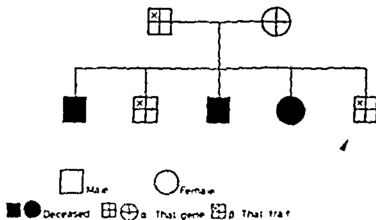


Fig 2 The family pedigree (the arrow indicates the propositus)

pattern but Hb A_2 values of the mother was def n tely decreased and the father s and brother s were elevated

Methods

Hemograms were obta ned by the standard techn ques Hem globin (Hb) con centrat ns were determ ned by the cyanmethemoglob n method and the m crohe matocr t was used f r measur ng packed cell volume Intraerythrocyt c inclus on sta n, aut hemolys s (w th and w thout glucose) and erythrocyt c osmot c frag l ty were d ne as described by DACIE and LEWIS [3] and the s ckl ng test by us ng 2% sod um metabisulf te Serum iron total iron bind ng capac ty and plasma hemogl bin c ncentrat ons were determ ned accord ng to SIMMONDS [4] PETERS et al [5] and CROSBY and FURTH [6] respect vely Starch gel and agar gel ele trophores s were performed by the methods of SMITHIES [7] and ROBINSON et al [8] succes s vely Hb F values were measured by the alkal denaturat on method of SINGER et al [9] and quant tat ve est mation of Hb A_2 was obta ned by chromatography on d ethylam noe hyl cellulose [10]

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evidence of the presence of α thalassemia in the father, who has α-β-thalassemia

To produce a child with Hb H disease, the mother must have the α-thalassemia gene. This was confirmed by the inclusion bodies in her erythrocytes. Her decreased Hb A_2 level can be related to α thalassemia since iron deficiency anemia is ruled out. The elevated Hb A_2 level in the patient's brother could indicated β thalassemia trait. Because of the rare inclusion body in his smear, we believe that he, like his father has α-β-thalassemia. Although Hb A_2 can be normal or decreased in patients with α-thalassemia, it is interesting that the father's and the brother's Hb A_2 level was around 5%, corresponding to reported values of Greek [16, 17], Chinese [18, 19], Thai [20], and Italian [19] double heterozygous persons. Hb A_2 elevation in Negro α-β-thalassemia cases was equivocal [21]. As FESSAS [16] has pointed out, no additive effect of these 2 (α+β) genes was observed in father's and brother's hematologic findings.

WASI *et al.* [20] have observed patients with major hemoglobin types of Bart's and A in whom evidence suggests the presence of the β thalassemia gene in addition to double α-thalassemia with the clinical picture of thalassemia intermedia. In view of the hypothesis of multiple α-chain loci for human hemoglobins [22], we prefer the theory postulated by LEHMANN [23, 24] to explain the variety of expression of α-thalassemia in these families. Using Lehmann's theory, not only the hemoglobin picture of the propositus, but the clinical severity of the disease in this family (as indicated by the death of 3 siblings apparently from the same disorder in early infancy and the persistence of chronic severe hemolysis in the propositus) be differentiated from the cases of WASI *et al.* [20]. Then, as indicated by the family pedigree (fig. 2), our patient had 3 α-thalassemia genes, and the patients of WASI *et al.* [20] probably had 2. If the relative rate of α- and β-chain synthesis in his and his parents' reticulocytes could be estimated by the extent of incorporation of [^{14}C]-L-leucine studies [17, 19], the extent of α-β-imbalance would be more definite.

The difference in the clinical expression of the disease in this family, may also be explained by the heterogeneity of β-thalassemia, but so far no supporting evidence could be obtained.

Acknowledgements. We are grateful to Dr. P. MIESNER and Dr. D. WEATHERALL for their helpful discussion and Mrs. B. ERDZAN for editorial help.

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Transplantation Reviews, vol 11 and 12 Munksgaard Copenhagen 1972 173 and 132 pp, dKr 135 – and 110 –

In vitro lymphocyte stimulation is a widely used tool for genetic studies, basic investigations concerning lymphocyte physiology and for appreciation of immune reactivity in man Many reviews have been written already on these topics in the past but progress is fast and far reaching Therefore, the two new volumes of Transplantation Reviews (11 and 12) dealing with lymphocyte stimulation are very welcome Both volumes contain reviews and progress reports by leading investigators in the field with the main emphasis on their own work

Volume 11 [illegible] llects seven papers on Lymphocyte activation by unspecific mitogens' The [illegible] paper covers RNA metabolism following activation (H L Cooper) follow[illegible] by a report on certain morphological and functional aspects of activation in ma[illegible] S D Douglas) The remaining five papers treat the large field of differential st[illegible] n and cooperation of T and B cells in relation to membrane receptors for [illegible] and the events leading to the stimulation (J D Stobo M Greaves e[illegible] Anderson *et al*, R T Smith P Baetscher) Each group reports on its w[illegible] ries to conclude on models for immunocyte activation in general The stu[illegible] papers is highly instructive and stimulating

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T L Vischer *Geneva*

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the authors After an intro[illegible]

P Stites and A C Wang **Basic Immunogenetics** London 1972 214 pp, £ 2.25

[illegible] es among individuals are often studied by [illegible] differences are expressed on the immuno[illegible] f lymphocytes It became clear during the [illegible] of immunoglobulins and lymphocytes [illegible] tivity Thus the field of immunogenetics [illegible] that the eminent geneticists from San [illegible] task to write a short new textbook [illegible] give not only an overall review of the [illegible] they stimulate the reader and give [illegible] nd basis Certain areas, of course [illegible] he personal work and experience of [illegible] structure and evolution of immuno[illegible]

lobulins are treated This is followed by the genetics of the immunoglobulin nolecules and the basis for antibody variability The fifth chapter on cells, genes ind the immune response reviews one of the most exciting and fast moving parts of immunogenetics Finally a long chapter is devoted to the human blood groups

The book is easy to read clear stimulating and can be recommended to any body with interests in biology T. L. VISCHER *Geneva*

Paroxysmal Nocturnal Hemoglobinuria (PNH) Series Haematologica vol V/1 Munksgaard Copenhagen 1972 175 pp dKr 110 –

Dieser Band fasst in glanzender Weise alte und neueste Erkenntnisse dieses faszinierenden Krankheitsbildes zusammen Er gibt eine gute Übersicht über die klinischen Verlaufsformen der PNH Übergange in aplastische Anamie akute Leukämie und Myelofibrose Diagnostische Tests werden *in extenso* besprochen Biochemische und funktionelle Storungen der PNH Zellen aller Linien werden in übersichtlicher Weise dargelegt Alte und neue therapeutische Massnahmen werden kritisch analysiert Dieses Übersichtswerk an dem viele der grossten Kenner des Krankheitsbildes mitgearbeitet haben kann jedem hamatologisch tatigen Arzt und jeder Bibliothek warmstens empfohlen werden B SPECK *Basel*

globulins are treated. This is followed by the genetics of the immunoglobulin molecules and the basis for antibody variability. The fifth chapter on cells, genes and the immune response reviews one of the most exciting and fast moving parts of immunogenetics. Finally, a long chapter is devoted to the human blood groups.

The book is easy to read, clear, stimulating and can be recommended to anybody with interests in biology. T. L. Vischer, *Geneva*

Paroxysmal Nocturnal Hemoglobinuria (PNH). Series Haematologica, vol. V/1. Munksgaard, Copenhagen 1972. 175 pp. dKr 110.–

Dieser Band fasst in glänzender Weise alte und neueste Erkenntnisse dieses faszinierenden Krankheitsbildes zusammen. Er gibt eine gute Übersicht über die klinischen Verlaufsformen der PNH, Übergänge in aplastische Anämie, akute Leukämie und Myelofibrose. Diagnostische Tests werden *in extenso* besprochen. Biochemische und funktionelle Störungen der PNH-Zellen aller Linien werden in übersichtlicher Weise dargelegt. Alte und neue therapeutische Massnahmen werden kritisch analysiert. Dieses Übersichtswerk, an dem viele der grössten Kenner des Krankheitsbildes mitgearbeitet haben, kann jedem hämatologisch tätigen Arzt und jeder Bibliothek wärmstens empfohlen werden. B. Speck, *Basel*

Acta Haemat. 50: 193-199 (1973)

Studies on Bone Marrow Transplantation in Experimental ^{32}P-Induced Aplastic Anemia after Conditioning with Antilymphocyte Serum

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Abstract. Aplastic anemia was produced with ^{32}P in rabbits with and without splenectomy. Transient hemopoietic engraftment without graft versus host (GvH) was seen after conditioning with antilymphocyte serum (ALS) and infusion of allogeneic marrow from a strain that is known to cause fatal secondary disease if total body irradiation is used for conditioning. More takes could be achieved in the splenectomized group but in both groups the takes were only transient. The absence of GvH was shown to be probably due to a split chimerism of the immunologic system. The graft rejections are felt to be due to the persistence of host T cells. Possibly the duration of engraftment can be prolonged in the future by continued immunosuppressive therapy after transplantation with ALS or cyclophosphamide. The fact that the spleen is not necessary for hemopoietic recovery after i.v. marrow infusion may have important clinical implications because many patients with aplastic anemia are splenectomized in the course of their disease.

Key Words
Antilymphocyte serum
Aplastic anemia
Bone marrow transplantation
Splenectomy

Despite modern therapeutic developments such as the use of anabolic steroids, transfusion of HL-A compatible platelets and granulocytes and nursing in a sterile environment, the prognosis of aplastic anemia (AA) is still very poor. Of 36 patients with this disease that we have seen on our Department of Hematology since 1967, two thirds have died and it has to be anticipated that even more of the patients with persisting pancytopenia in this series will have a fatal outcome.

It has been clearly demonstrated that bone marrow transplantation can be successful in AA after conditioning with cyclophosphamide (Cy) if

an HL-A identical sibling is available as marrow donor [1, 2, 3]. However, most of the patients are not in the fortunate situation of having an HL-A identical brother or sister. MATHÉ *et al.* [4] were the first to show that takes can be achieved and graft versus host (GvH) avoided if antilymphocyte serum (ALS) alone is used for conditioning of these patients, even if the donor is not HL-A identical. We have confirmed this finding in rabbits with benzene induced aplastic anemia [5, 6]. The present study deals with ^{32}P induced marrow aplasia and bone marrow transplantation after conditioning with ALS.

Material and Methods

Marrow aplasia was induced in 40 male rabbits of a cross between Vienna White and Alaska of 1–2 kg. Group 1, consisting of 20 animals, was given 1.2 mCi/kg ^{32}P. Ten of these rabbits were given marrow grafts after conditioning with ALS. Five were transplanted without conditioning and 5 were not grafted. Group 2, consisting of 20 animals, was given 1.4 mCi/kg ^{32}P 10 days after they had been splenectomized. Ten of them were grafted after conditioning and 5 were not transplanted.

Conditioning was performed with 3ml/kg ALS subcutaneously on 4 subsequent days. *ALS was produced in a horse by repeated injections with rabbit thoracic duct* lymphocytes. The efficacy of this serum was clearly established by prolongation of skin allograft survival and by *in vitro* inhibition of rosette formation [7]. Conditioning with ALS was started as soon as the peripheral white blood cell count had dropped below 1,500 mm^3. This was usually 2–3 weeks after the ^{32}P. Marrow grafts were given 24h after the last dose of ALS. Female Dutch rabbits of 0.8–1.2 kg served as marrow donors. 2–3 × 10^8/kg nucleated bone marrow cells were given intravenously. Previous marrow transplants between these 2 strains have been *uniformly followed by fatal secondary disease if total body irradiation (TBI)* was used for conditioning [SPECK, KISSLING, BOORMAN and HOLLÄNDER, unpubl. data].

Follow-up studies. In all rabbits weekly blood counts, including hemoglobin, leukocytes, differential and platelets were performed. Cytogenetics of the bone marrow cells were performed as soon as there was any evidence of red cell *chimerism. They were repeated if the chimerism lasted more than 8 weeks.* A modified short term culture method of MOORHEAD [8, 9] was used. The proportion of XX and XY chromosomes were quantitated in at least 10 metaphases. Erythro*cyte typing for the antigens A, D and F was performed every 1–2 weeks according* to the method described by COHEN [10]. Drumsticks were looked for in the peripheral granulocytes once weekly. Immunoglobulin allotype studies were performed in the donor and recipient before grafting at 3- to 4-weekly intervals afterwards [11]. First and second set skin allograft survival studies from the previous marrow donor were performed on two long term chimeras. Two-way mixed leukocyte cultures between a long term chimera and the previous marrow donor were studied on day 80. Autopsies, histopathologic and bacteriologic studies were performed on all animals that died.

Table I. Course of control group not conditioned with ALS

	Number of animals	Died	Survival weeks	Cause of death		Hemopoietic recovery
				hemorrhage	sepsis	
No splenectomy ^{32}P only	5	3	4 (2.5–5.5)	2	1	2
No splenectomy ^{32}P plus marrow	5	5	3.8 (3–5)	3	2	–
Splenectomy ^{32}P only	5	3	5.5 (3.5–6)	1	2	2
Splenectomy ^{32}P plus marrow	5	3	5 (4–6)	–	3	2

14 animals died between 2.5 and 6 weeks. No evidence of donor marrow engraftment could be demonstrated in the 2 groups that were transplanted.

Results

Hematologic changes consisted mainly of thrombocytopenia and granulocytopenia which were noted in the second week after ^{32}P. The absolute lymphocyte and hemoglobin counts decrease only minimally.

In the control group the ^{32}P was not uniformly fatal, and 6 spontaneous hemopoietic recoveries occurred: 4 times in animals that had ^{32}P only and twice in animals that had a marrow graft. In the latter there was never any evidence of donor marrow engraftment. 14 control rabbits died between 2½ and 6 weeks after ^{32}P either from septicemia or hemorrhage (table I).

Rabbits that were conditioned with ALS showed a high percentage of transient donor marrow engraftment (table II). Evidence of the take was seen between the third and fifth week after grafting. Karyotyping of the bone marrow cells usually showed between 20 and 40% of the cells to be of donor origin, but never more than 50%. Red cell typing with various dilutions of the antisera never indicated complete red cell chimerism. Drumsticks in the granulocytes were only occasionally found, but never reached the 2–3% that we had established to be normal for female rabbits of this strain. Immunoglobulin allotype markers were present in 5 grafts. In 2 of them they turned to donor type after the third week and did not change any more during the follow-up period of over 10 weeks.

Table II. Findings in the rabbits that were conditioned with ALS before bone marrow transplantation

	Number of animals	Evidence of take	Duration of take weeks	No take	Died too early for evaluation
1. Not splenectomized	10	4	7.5 (5–13)	2	4
2. Splenectomized	10	8	7 (5–12)	1	1

In splenectomized animals a much higher percentage of takes could be achieved, which may be due to the fact that in group 1, many animals died within a few days with severe pancytopenia. The duration of takes is comparable in both groups. In all the 12 animals that had transient takes of the donor marrow, their own hemopoiesis recovered and they became long term survivors.

First set skin allografts from the previous marrow donor were rejected between 10 and 12 days and second set rejections occurred within 7 days. The same first and second set rejection times were found in 4 normal controls of the same sex and strain. Also, the MLC between marrow donor and recipient was positive.

None of the animals developed any evidence of GvH. They behaved normally and gained weight normally. The animals dying during the course of the experiment were subjected to careful histological studies which did not yield any evidence of GvH.

Discussion

In ³²P induced aplastic anemia in rabbits, a high percentage of takes of histo incompatible bone marrow can be achieved after conditioning with ALS. As in the previously reported rabbits with benzene induced aplastic anemia [5, 6], GvH is completely absent which in both experimental models is probably due to the fact that T cells remain of host type. The major difference between the two models was that the duration of marrow engraftment was much shorter in the ³²P induced aplasias. Here the first rejections occurred after 5 and the latest after 13 weeks. In the

benzene induced aplasias only one out of 6 animals that did have takes rejected the graft in the observation period of over 15 weeks.

The chimerism documented with cytogenetics of the bone marrow cells, red cell antigens and drumsticks, was much less complete in the ^{32}P than in the benzene induced aplasias. The chimerism of the immunologic system is comparable in both models. Unfortunately, immunoglobulin allotype markers were only present in 5 grafts. In 2 instances the allotypes turned to donor type after the third week and remained entirely donor during the follow up period of over 10 weeks. This implies a take of donor B cells. In the other 3 grafts there was either no take of B cells, or else the method was not sensitive enough to detect small amounts of donor immunoglobulin allotypes. The presence of host T cells during the chimeric state has been established by normal first and second set skin allograft rejections from previous marrow donor as well as with a positive MLC between donor and recipient.

It was very striking that animals that were previously splenectomized were much easier to transplant. Despite the fact that they had higher doses of ^{32}P, they developed less pronounced pancytopenia and also they tolerated the ALS better. This probably explains why more splenectomized animals survived the immediate post transplantation period. It is a well known fact of radiobiology that splenectomized animals tolerate higher doses of TBI [12] and chemotherapists know that splenectomized patients tolerate higher doses of cytostatic agents. The fact that hemopoietic recovery after intravenous bone marrow infusion can occur in rabbits without a spleen is very interesting and may have important clinical implications because many patients with AA are splenectomized in the course of their disease.

In 2 experimental models of AA, we have documented that after conditioning with ALS alone the host T cells are temporarily suppressed, which probably allows for a take of the donor marrow. Later the host T equivalent returns which may explain the absence of GvH and the frequent graft rejections. This has been confirmed in clinical patients with AA conditioned with ALS. GvH was absent and the take limited to erythropoiesis, myelopoiesis, thrombopoiesis and in several instances the B equivalent of the immunologic system [13, 14]. Special emphasis has to be placed on the fact that all these takes were transient lasting a maximum of 20 months [13]. The place of ALS for conditioning patients with AA for bone marrow transplantation has as yet to be definitely established. Great advantages are that fatal GvH can be avoided even if the do-

nor is not an HL-A identical sibling However, the number of takes appears to be smaller than after conditioning with Cy and only of transient nature [13] On the other hand, they may suffice for spontaneous recovery of the patient's own hemopoietic system In addition, new approaches for prevention of early graft rejection might be helpful possibly continued T cell suppression with ALS or Cy after transplantation

Acknowledgements We wish to thank Prof D W VAN BEKKUM for his help for the production of ALS Prof J J VAN ROOD for providing laboratory and animal facilities and his critical comments on the manuscript, Prof R B EPSTEIN for suggesting ³²P to induce marrow aplasia Dr A S KELUS for the immunoglobulin allotype determinations and Drs. C F HOLLANDER and G A BOORMAN for their pathologic anatomic studies.

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Acta Haemat. 50: 200–212 (1973)

Säulenchromatographische Anreicherung von DNA-Polymerase-Aktivitäten bei Leukämie

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Abstract. Leukemic leukocytes revealed 2 species of DNA polymerases: DNA-dependent and RNA-dependent polymerase activities. The template characteristics of the RNA instructed enzymes were similar to those found with the DNA polymerase of oncogenic RNA viruses. Some minor differences in the molecular weight of RNA instructed DNA polymerase activities could be taken as arguments for some heterogeneity of reverse transcriptase in different forms of leukemia. The most striking difference of these results is to the DNA polymerase content of normal leukocytes or blast cells, inasmuch as normal blood cells lack RNA-dependent DNA polymerase activities. The possible role of reverse transcriptase in transforming cells led to the search for inhibitors. Therefore, some rifamycins and distamycins have been tested for the influence on DNA instructed DNA synthesis in a cell free system.

Key Words
Chromatography
DNA polymerases
Leukemic cells
Rifamycins

Nach bisher vorliegenden Literaturangaben erscheint es, dass die Umkehr des Flusses der genetischen Information, also die Übertragung von RNA auf DNA durch das Enzym reverse Transkriptase in RNA-Viren spezifisch für Onkogenität [6] und in eukaryoten Zellen nur in embryonalen oder Tumorzellen aufgefunden werden kann [1, 3–8].

Die Ätiologie der menschlichen Leukämie ist bisher unbekannt. Aus den verschiedensten Überlegungen wird jedoch ein onkogenes RNA-Virus als mögliche Ursache dieser Erkrankung diskutiert [4–8]. Die Suche nach RNA-Viruspartikel in Leukämiezellen ist bisher jedoch durchwegs negativ verlaufen. Da offensichtlich der Virusnachweis mit den heutigen Methoden noch nicht gelingt, könnte der Nachweis eines

viralen Enzyms mit den chemischen Eigenschaften des Temin-Baltimore-Enzyms [2, 17] hierfür eine sensitivere Nachweismethode darstellen

Material und Methoden

2-[14]C-Thymidintriphosphat (spez. Akt. 32 mCi/mM) wurde vom Radiochemical Centre Amersham bezogen, Poly rA.dT, Poly d (A-T), rA.dT$_{10}$ und dA.dT$_{10}$ von Miles, DEAE-Zellulose GS, Zytochrom C, MG 12 400, Chymotrypsinogen A, MG 25 000, Rinderalbumin, MG 67 000 und Aldolase, MG 160 000, von Serva, Phosphozellulose von Whatman, Nonidet von Shell, dATP, dCTP, dGTP, Dithiotreitol und Sephadex G 200 von Sigma. Das Distamycinderivat Nr. 152/30 wurde uns von der Firma Farmitalia zur Verfügung gestellt. Die Rifamycinderivate 45623-Ba, 45733-Ba und 18414-Ba wurden uns von der Ciba-Geigy überlassen. Alle übrigen Reagentien waren P.a.-Qualität und stammten von Sigma oder Merck.

Die Blutzellen wurden von Patienten mit akuter Leukose (AL, POX-Typ), chronisch myeloischer Leukämie (CML) und chronisch lymphatischer Leukämie (CLL) gewonnen. Bei allen Spendern wurde die klinische Diagnose durch Sternalpunktion und histochemische Spezialfärbungen gesichert. Die für unsere Untersuchungen herangezogenen Patienten waren entweder unbehandelt oder hatten während der letzten 3 Monate vor der Entnahme von Blutzellen keine Zytostatika oder Glukokortikoide erhalten. Die Gewinnung der Blutzellen erfolgt mit der Zellzentrifuge von Aminco. Die entnommenen Zellzahlen schwankten zwischen 10^{9} und $8 \cdot 10^{10}$ Leukozyten.

1. Präparation der DNA-Polymerasen aus leukämischen Leukozyten. Die unter sterilen Kautelen gewonnenen Leukämiezellen wurden 10 min bei 60 g zentrifugiert und das überstehende Plasma abgesaugt. Das resultierende Zellpellet wurde in 2 vol. Phosphatpuffer (0,01 m, pH 7,4, 20% Glycerin, 0,001 m EDTA, 0,001 m Dithiotreitol [DTT], 0,5% Na-deoxycholat, 2% Nonidet) suspendiert und im Braun-Mixer homogenisiert. Das Homogenat wurde 20 min bei 6000 g und der resultierende Überstand bei 105 000 g 1 h zentrifugiert.

2. Anreicherung der DNA-Polymerasen auf DEAE-Zellulose. Puffer A: 0,05 m Phosphatpuffer, pH 7,2, 2 mM DTT, 10% Glycerin. Puffer B: 0,3 m Phosphatpuffer, pH 7,2, 2 mM DTT, 10% Glycerin. Puffer C: 2 mM DTT, 10% Glycerin.

200 g DEAE-Zellulose wurden auf einem Büchner-Trichter mit 3 l Puffer A äquilibriert. Anschließend wurde der 105 000-g-Überstand 10fach mit Puffer C verdünnt und auf die Zellulose aufgetragen, mit 5 l Puffer A nachgewaschen und eine Proteinfraktion mit 3 l Puffer B eluiert. Die Waschlösung wurde 3fach, die Elutionslösung 6fach mit Puffer C verdünnt. Das pH der beiden Lösungen wurde mit KOH auf 8,0 eingestellt.

3. Weitere Anreicherung von DNA-Polymerasen durch Chromatographie auf Phosphozellulose. Puffer A: 0,005 m Phosphatpuffer, pH 8,0, 2 mM DTT, 10% Glycerin. Puffer B: 0,6 m Phosphatpuffer, pH 8,0, 2 mM DTT, 10% Glycerin.

Zwei 4 cm messende Säulen wurden 1 m hoch mit Phosphozellulose gefüllt und mit 2 vol. Puffer A äquilibriert. Auf eine Säule wurden die unter Punkt 2 erhaltene Waschlösung und auf die andere Säule die Elutionslösung aufgetragen. Die

Elution der DNA-Polymerasen erfolgte mit einem linearen Phosphatpuffergradienten (je 2 l Puffer A, Puffer B) und mit Hilfe einer peristaltischen Pumpe. Die Flussrate betrug etwa 0,7 ml/min. Es wurden Fraktionen zu ungefähr 20 ml gesammelt und auf Polymeraseaktivität ausgetestet. Die Gipfelfraktionen wurden gepoolt und im Amicon-Ultrafiltriergerät konzentriert (PM 10 Filter).

4. Ermittlung des Molekulargewichtes durch Gelfiltration auf Sephadex G 200. 35 g Sephadex G 200 wurden 3 Tage in 0,15 *m* Phosphatpuffer (pH 8,0), 2 mM DTT und 10% Glycerin quellen gelassen, und anschliessend wurde eine 5 cm im Durchmesser messende Säule 1,3 m hoch gefüllt. Nach Äquilibrieren mit 2 Säulenvolumina Phosphatpuffer wurden die Enzymlösungen aufgetragen. Es wurden Fraktionen zu etwa 20 ml gesammelt. Als Referenzverbindungen wurden Dextranblau, Zytochrom C, MG 12 400, Chymotrypsinogen, MG 25 000, Rinderalbumin, MG 67 000, und Aldolase, MG 160 000, analog chromatographiert.

5. Bestimmung der Polymeraseaktivität. In 100 µl Testansatz waren enthalten: 50 mM Tris-HCl, pH 7,8, 80 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 0,08 mM TTP, 15 µl TTP-^{14}C, je 0,08 mM dATP, dCTP, dGTP, 10 µg Rinderalbumin und 0,5 µg Template-NA. Zu der angegebenen Mischung wurden 20 µl Enzymlösung zugesetzt und das Inkubationsgemisch 30 min bei 37 °C inkubiert. Nach Abschluss der Inkubationsperiode wurden 100 µl Anteile auf Whatman-3MM-Filterpapierchen aufgetragen, die Plättchen getrocknet und auf einem Büchner-Trichter in der Reihenfolge mit den folgenden Lösungen je 2× gewaschen (zirka 200 ml): 5% Trichloressigsäure (TCA), 5% TCA + 1% Tetranatriumpyrosulfat, 5% TCA, Äthanol, Äther. Nach dem Trocknen wurden die Plättchen mit 10 ml Toluolszintillator (6 g Butyl-PBD/1 l Toluol) im Tri-Carb (Packard) gezählt. Alle Ergebnisse wurden auf einen mit diesem Versuchsansatz ermittelten Blindwert korrigiert.

6. Beeinflussung der ^{14}C-TTP-Inkorporation durch Zusatz von Distamycinderivaten bzw. Rifamycinderivaten in den Testansatz zur Bestimmung der Polymeraseaktivität. Die Distamycin- oder Rifamycinderivate wurden in Dimethylsulfoxyd (DMSO) gelöst und dem unter Punkt 5 beschriebenen Testansatz zugegeben. Blindwert und Leerwert erhielten lediglich den Zusatz einer adäquaten Menge DMSO.

Ergebnisse

Das Elutionsprofil der DNA-abhängigen DNA-Polymerasen wurde durch Austesten der einzelnen Fraktionen mit d(A-T) oder dA·dT$_{12}$ ermittelt, das der RNA-abhängigen DNA-Polymerasen mit den Templates rA·dT bzw. rA·dT$_{12}$ [6, 7, 9, 14, 16].

1. Chromatographisches Verhalten der D-DNA-Polymerasen aus leukämischen Zellen. Die an DEAE-Zellulose (0,05—0,3 *m* Phosphatpuffer) vorgereinigten Enzyme wurden auf Phosphozellulose aufgetragen und mit Hilfe eines Phosphatpuffergradienten eluiert. Die einzelnen Fraktionen wurden gesammelt und auf Polymeraseaktivität getestet. Diese Enzympräparationen waren offensichtlich weitgehend frei von

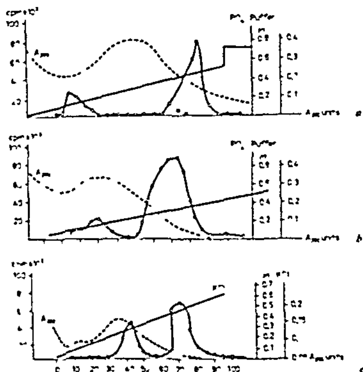


Abb. 1. Chromatographisches Verhalten der DNA-Polymerasen menschl. Leukämiezellen nach Vorreinigung an DEAE-Zellulose (0,05–0,3 m Phosphatpuffer) und anschließender Chromatographie auf Phosphorzellulose. Die Polymerasen wurden von der Phosphorzellulose durch Anlegen eines linearen Phosphatpuffergradienten eluiert. Es wurden Fraktionen zu etwa 20 ml gesammelt und Anteile von 20 µl jeweils auf Polymeraseaktivität mit d(A-T) als Template angetestet. Ordinate: cpm/20 µl Enzym. Elutionsprofile der D-DNA-Polymerasen bei CLL *(a)*, CML *(b)* und AL, POX Typ *(c)*

Nukleinsäuren mit der Diphenylaminreaktion konnte kein DNA-Gehalt nicht nachgewiesen werden. Die Abbildungen 1a und 1b zeigen das Elutionsprofil der D-DNA-Polymerasen, die von Patienten mit CLL bzw. CML gewonnen wurden. Die beiden Chromatographien erscheinen nahezu identisch. Abbildung 1c stellt das chromatographische Verhalten der D-DNA-Polymerasen bei AL dar. Es zeigt ebenfalls 2 Peaks, die nach ihrer Elutionsfolge von Phosphorzellulose von GALLO als Polymera-

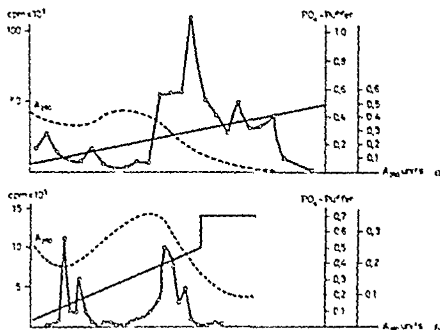


Abb. 2 Chromatographisches Verhalten der R-DNA-Polymerase-Aktivitäten aus menschlichen Leukämiezellen nach Vorreinigung an DEAE-Zellulose (0,05–0,3 m Phosphatpuffer) und anschliessender Chromatographie auf Phosphozellulose. Die Polymerasen wurden von der Phosphozellulose durch Anlegen eines linearen Phosphatpuffergradienten eluiert. Es wurden Fraktionen zu zirka 20 ml gesammelt und Anteile von 20 µl mit rA·dT als Template auf DNA-Polymerase-Aktivität getestet. Ordinate: cpm/20 µl Enzym. Abszisse: Fraktionsnummern. Elutionsprofile der R-DNA-Polymerasen bei CLL (*a*) und CML (*b*).

se I und Polymerase II bezeichnet werden. Gegenüber den Abbildungen 1a und 1b fällt auf, dass die Polymerase I geringfügig höher ist und erst mit hohermolarer Salzkonzentration eluiert werden kann.

2. *Chromatographisches Verhalten der R-DNA-Polymerase-Aktivitäten aus leukämischen Zellen.* Abbildung 2a zeigt das chromatographische Verhalten der R-DNA-Polymerase-Aktivitäten aus CML, Abbildung 2b bei CLL. Ähnliche Profile wurden auch bei AL erhalten. Gemeinsam war allen 3 Leukämieformen, dass sich mit den Templates rA·dT und rA·dT$_{18}$ durchwegs eine mehrgipfelige Kurve ergab. Man könnte fälschlicherweise vermuten, dass es sehr viele verschiedene reverse Transkriptasen in Leukämiezellen geben könnte. Um diese Frage näher abzuklären, wurden alle Gipfelfraktionen einer Sephadexchroma-

Tabelle I. Beeinflussung der [14]C-TTP-Inkorporation durch Zugabe von Distamycinderivaten (Substanz 182 = C XIII) bzw. Rifamycinderivaten (Substanz 45723-Ba = C 11, 45414-Ba = C 22, 45733-Ba = C 27) in den Testansatz zur Bestimmung der Enzymaktivität der Polymerasen I und II aus leukämischen Leukozyten

Substanz	Menge µg	Polymerase I %	Polymerase II %
Kompletter Ansatz		100	100
C XIII	5	36	84
	10	14	34
C 11	5	96	102
	10	270	190
	15	930	820
C 22	5	87	93
	10	360	270
	15	860	1002
C 27	5	113	107
	10	470	240
	15	890	810

tographie unterworfen und die mit dieser Chromatographie erhaltenen Peaks auf Templatecharakteristika untersucht. Wie in Tabelle II gezeigt wird, erfüllt jedoch lediglich ein Gipfel alle Voraussetzungen für eine reverse Transkriptase. Die Ursache für diese vielgipfeligen Kurven liegt also lediglich darin, dass uns kein »spezifisches« Template für die reverse Transkriptase zur Verfügung stand und aus diesem Grunde viel schlechtere Parameter für den Enzymnachweis berücksichtigt werden mussten [3–8, 14, 16].

3. *Beeinflussung der D-DNA-Polymerasen I und II durch Zusatz von Rifamycin- und Distamycinderivaten.* Für diese Untersuchungen wurden Substanzen gewählt, welche bekanntlich die RNA-abhängige DNA-Polymerase onkogener RNA-Viren hemmen [5, 6, 10]. Während Rifamycinderivate eher eine Aktivierung der D-DNA-Polymerasen leukämischer Zellen herbeiführten, bewirkte Distamycin eine Hemmung der beiden D-DNA-Polymerasen (Tab. I). Die Hemmwirkung der untersuchten Substanzen auf die R-DNA-Polymerase konnte bestätigt werden.

4. *Molekulargewichtsbestimmung der D-DNA- und R-DNA-Polymerasen auf Sephadex G 200.* Die Peakfraktionen der Phosphocellulosechromatographie wurden gepoolt und auf eine Sephadex-G-200-Säule aufgetragen. Das Ergebnis, welches für die lymphatische Leukämie erhalten

Tabelle II Template-(Primer-)Charakteristika von DNA-Polymerase-Aktivitäten bei Leukämie (TTP-Inkorporation in pMol/h/µg Protein). Die in der Sephadexchromatographie bereiteten Fraktionen Polymerase I, Polymerase II und reverse Transkriptase wurden gesammelt, eingeengt und mit den verschiedensten Templates auf DNA-Polymerase-Aktivität getestet

Template		DNA-Polymerase		
		I	II	Reverse
$dT_{10} \cdot rA$	Mg^{++}	12	0	361,00
$dT_{10} \cdot rA$	Mn^{++} (-Mg)	23	10	472,00
$dT_{10} \cdot dA$	Mg^{++}	97	5 400	320,00
Poly rA · dT	Mg^{++}	0	20	98,00
Poly rA · dT	Mn^{++} (-Mg)	56	40	125,00
Poly dA · dT	Mg^{++}	48	2 570	38,30
DNA nativ	Mg^{++}	2	670	13,00
DNA denat.	Mg^{++}	3	2 060	24,00
Poly rA · rU	Mg^{++}	3	0	290,00
Poly rA · rU	Mn^{++} (-Mg)	9	0	321,00
$tRNA^{Phe}_{E. coli}$	Mg^{++}	32	120	354,00
Q_β RNA + dT				
^{14}C TTP	Mg^{++}	10	50	325,00
Q_β RNA + dT				
^{3}H-dGTP	Mg^{++}	0	0	287,00

wurde, ist in Abbildung 3, das der myeloischen Leukämie in Abbildung 4 wiedergegeben. Bei beiden Leukämieformen konnten 2 D-DNA-Polymerasen nachgewiesen werden, welche analoges Verhalten hinsichtlich ihres Molekulargewichtes aufweisen. Die Gipfel der R-DNA-Polymerasen verhalten sich jedoch bei den von uns untersuchten Fällen verschieden. So besitzen die R-DNA-Polymerase-Aktivitäten aus myeloischer Leukämie ein wesentlich höheres Molekulargewicht als bei lymphatischer Leukämie. Besonders erachtenswert erscheint es dabei, dass die Chromatographie der R-DNA-Polymerasen eine Reihe von Fraktionen sowohl bei myeloischer wie bei lymphatischer Leukämie ergab, welche $rA \cdot dT_{18}$ weit besser als $dA \cdot dT_{18}$ ablesen können.

Das Molekulargewicht der DNA-Polymerase I beträgt etwa 150 000, das der Polymerase II etwa 30 000. Die Template-(Primer) Charakteristika werden in der Tabelle II wiedergegeben. Diese Untersuchungen wurden mit den Enzympräparationen der Sephadexchromatographie durchgeführt.

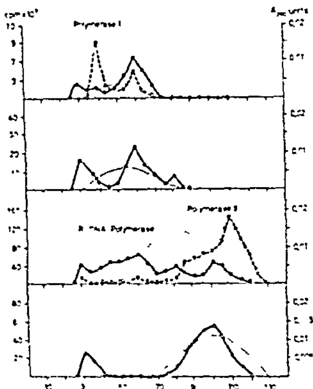


Abb. 3. Chromatographisches Verhalten der D-DNA-Polymerasen aus Abbildung 1a und der R-DNA-Polymerasen aus Abbildung 2a auf Sephadex G 200. Das Elutionsprofil der Polymerasen der Phosphocellulosechromatographie wurde den Peakfraktionen entsprechend in 4 Teile eingeteilt. Diese Gipfelfraktionen wurden gesammelt und durch Ultrafiltration auf ein Volumen von etwa 10 ml eingeengt (Amicon, PM 10-Filter). Die 4 Chromatographieanteile wurden in der entsprechenden Reihenfolge auf eine 1,2 m hohe und 3 cm im Durchmesser messende Chromatographiesäule aufgetragen und getrennt eluiert. Die Kurven stellen das Elutionsverhalten dieser Polymeraseaktivitäten nach Gelfiltration dar. Es wurden Fraktionen zu je 20 ml gesammelt und 20 µl Anteile auf Polymeraseaktivität mit den Templates rA·dT$_{12}$ (●) und dA·dT$_{12}$ (○) getestet. Ordinate: cpm/20 µl Enzym. Abszisse: Fraktionsnummern. —— = A$_{280}$ nm.

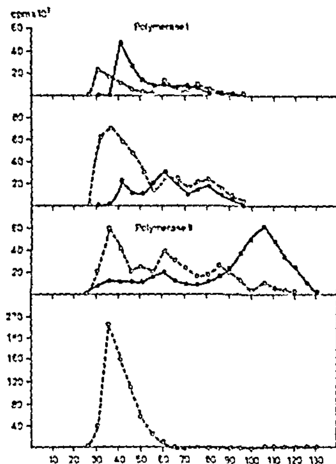


Abb 4 Chromatographisches Verhalten der Peakfraktionen der D DNA Polymerasen aus Abbildung 1b und der R DNA Polymerasen aus Abbildung 2b auf Sephadex G 200 Versuchsordnung siehe Legende zur Abbildung 3 ○ = R DNA Polymerase Aktivität ● = D DNA Polymerase Aktivität

Diskussion

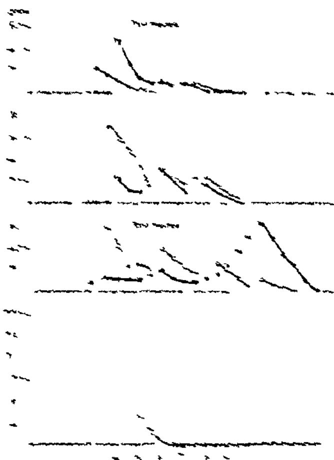
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trationen sämtliche in Leukämiezellen vorkommende Polymerasen zu extrahieren. Allerdings fanden wir im Gegensatz zu GALLO bei CML und CLL R-DNA-Polymerase-Aktivitäten, welche nach chromatographischen Kriterien verschiedene Enzyme sein könnten.

Ein bemerkenswerter Unterschied gegenüber normalen Blutzellen oder nicht leukämischen Blasten liegt jedoch darin, dass in leukämischen Zellen DNA-Polymerase-Aktivitäten beobachtet werden konnten, welche rA·dT$_{18}$ weit besser ablesen können als dA·dT$_{18}$ [1, 3–8, 14]. Wie wir nachweisen konnten, ist diese R-DNA-Polymerase auch in der Lage, das Template Qβ-RNA abzulesen. Die nachgewiesene Inkorporation in das säurefällbare Präzipitat mit ^{14}C-TTP und ^{3}HdGTP spricht in diesem Zusammenhang dafür, dass wirklich heteropolymere Anteile einer Ribonukleinsäure in eine Desoxyribonukleinsäure transkribiert wurden. Da die Qβ-RNA die Ribonukleinsäure eines RNA-Virus darstellt, haben wir in unserem Versuch demnach ein DNA-Provirus eines nicht onkogenen RNA-Virus im zellfreien System hergestellt.

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Die bisher gebräuchliche Behandlung der Leukämie mit ganz unspezifisch wirkenden Substanzen der Zellteilungshemmung mag unter dem Eindruck



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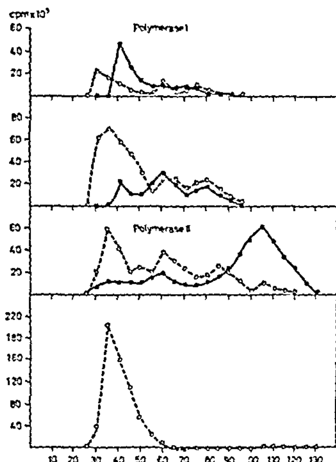


Abb 4 Chromatographisches Verhalten der Peakfraktionen der D-DNA Polymerasen aus Abbildung 1b und der R-DNA Polymerasen aus Abbildung 2b auf Sephadex G-200 Versuchsordnung siehe Legende zur Abbildung 3 ○ = R-DNA Polymerase Aktivität, ● = D-DNA Polymerase Aktivität

Diskussion

In unseren Untersuchungen wurden DNA-Polymerase-Aktivitäten chromatographisch hoher angereichert und fraktioniert Die Profile der D-DNA- und R-DNA-Polymerase-Aktivitäten bei AL, CLL und CML waren in unserer Versuchsanordnung nicht wesentlich unterschieden Die Ergebnisse stehen im Einklang mit den Befunden von GALLO [6], GALLO *et al* [5, 7, 8] und LOEB *et al* [15], obwohl wir bestrebt waren, durch Behandlung der intakten Zellen mit sehr hohen Detergenskonzen-

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der sich mehrenden Berichte über eine Störung der Zelldifferenzierung bei diesem Krankheitsbild als nicht therapeutisch zum Ziele führend erscheinen. Die Isolierung RNA-abhängiger DNA-Polymerasen aus Blutzellen [5, 6, 10] und onkogenen RNA-Viren wurde daher als *In-vitro*-Ansatz zur Auffindung antileukämischer Substanzen herangezogen. In der Zwischenzeit wurden verschiedenste Verbindungen ermittelt, welche die DNA-Polymerase der RNA-Viren hemmen [6, 10, 11]. In vielen Fällen wurde jedoch dabei ausser Betracht gelassen, dass diese Hemmstoffe möglichst spezifisch auf das virale Enzym wirken sollten, die normalen und zellständigen eukaryoten DNA-Polymerasen jedoch möglichst unbeeinflusst bleiben sollten. Die von uns untersuchten Rifamycinderivate scheinen dafür gewisse Voraussetzungen zu erfüllen und berechtigen zu weiteren Untersuchungen. Das Distamycinderivat mit der beobachteten Hemmwirkung auf DNA-abhängige DNA-Polymerasen erscheint dagegen als ein weit weniger spezifischer Hemmstoff. In diesem Zusammenhang ist es von Interesse, dass von verschiedenen Laboratorien über eine selektive Toxizität von Hemmstoffen der reversen Transkriptase auf transformierte Zellen berichtet wurde [8, 11].

Das Studium der DNA-Polymerasen normaler und leukämischer Zellen beansprucht daher in besonderer Weise experimentelles Interesse, um die pathophysiologischen Grundzüge der Leukämie besser verstehen zu können. Das Konzept, dass die in Leukämiezellen vorkommende reverse Transkriptase eine Rolle für das Initialstadium der Leukämie spielt und unter Umständen auch daran beteiligt ist, das Stadium der Malignität aufrechtzuerhalten, liesse daran denken, dass die Anwendung *von Hemmstoffen der RNA-abhängigen DNA-Polymerasen ein neues* therapeutisches Prinzip für die Behandlung maligner Erkrankungen darstellen könnte.

Diese Untersuchungen wurden mit Unterstützung des österreichischen Forschungsrates (Projekt 1477) und im Auftrag des österreichischen Bundesministeriums für Wissenschaft und Forschung durchgeführt.

Zusammenfassung

In leukämischen Leukozyten konnten 2 verschiedene DNA-Polymerase-Aktivitäten durch säulenchromatographische Auftrennung nachgewiesen werden: DNA-abhängige DNA-Polymerasen (Polymerase I und Polymerase II) und RNA-abhängige Polymeraseaktivitäten. Die Template-(Primer-)Charakteristika dieser RNA-

abhängigen DNA Polymerasen waren ähnlich wie für die reverse Transkriptase onkogener RNA Viren Gewisse Unterschiede im Molekulargewicht RNA abhängiger DNA Polymerasen bei lymphatischer Leukämie gegenüber myeloischer Leukämie sprechen für eine gewisse Heterogenität dieser Enzyme bei verschiedenen Leukämiespezies. Wesentlich ist der Unterschied zu normalen Blutzellen oder nicht leukämischen Blasten, da in diesen Zellen bisher niemals eine reverse Transkriptase nachgewiesen werden konnte. Die mögliche Beteiligung dieses Enzyms bei den Vorgängen der Malignität hat uns daher veranlasst, in einem *In vitro*-screening System verschiedene Hemmstoffe der reversen DNA Polymerasen auf die Beeinflussung der DNA instruierten DNA Synthese zu untersuchen

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Acta Haemat. 50: 213–216 (1973)

Le syndrome de Richter

Rapport de quatre observations et essai de démembrement

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Fondation Bergonié, Bordeaux

Abstract. On the basis of 4 personal observations and a review of the literature the authors undertake an analysis of Richter's syndrome. Sometimes chronic lymphoid leukemia (CLL) and malignant reticulopathy (MR) are discovered simultaneously; sometimes MR is diagnosed after one or more years of development of a known CLL. In the latter case the appearance of the MR may be accompanied by persisting signs of the CLL, by their regression disappearance. These differing situations probably have different meanings.

Key Words
Hodgkin's disease
Lymphatic leukaemia
Reticulosarcoma
Richter's syndrome

Depuis son individualisation par LORTHOLARY *et al.* [1], le syndrome de Richter, associant une leucémie lymphoïde chronique (LLC) et une reticulopathie malig[illegible] (reticulosarcome ou maladie de Hodgkin), a fait l'objet de quelques [illegible]cations rapportant de nouvelles observations en envisageant quelques [illegible]lications pathogeniques.

Ayant récemmen[illegible] [illegible] quatre sujets atteints de cette maladie, il nous est apparu q[illegible] appellation recouvrait en realité des entites diverses, méritant d'[illegible] [illegible]vidualisées et repondant probablement à des pathogenies differente[illegible] ce que nous discuterons brièvement après avoir rapporté nos q[illegible] [illegible]servations personnelles qui illustrent cette diversité.

[illegible]rvations

Observation 1. Mlle B[illegible] des adénopathies cervicales [illegible] [illegible] Un traitement par [illegible] remission stable et de bonne [illegible]

[illegible] decouvre à l'âge de 45 ans, en 196[illegible] [illegible] à faire un diagnostic de LLC [illegible] appliqué pendant six mois, entraîne une [illegible] fevrier 1970 l'etat général de la malade

présente une altération brutale avec fièvre et compression médiastinale d'origine ganglionnaire avec une polyadénopathie superficielle discrète. On constate alors une disparition de tout signe de LLC dans le sang comme dans la moelle et on évoque un syndrome de Richter qui est confirmé par une biopsie ganglionnaire cervicale affirmant un lymphoréticulosarcome. Il s'agit d'un stade III$_B$ qui fait l'objet d'une polychimiothérapie réductrice puis d'une irradiation en deux temps de toutes les aires ganglionnaires tronculaires, suivie d'une chimiothérapie d'entretien par vincristine. La malade reste en rémission apparemment complète quand elle décède en mars 1972 de décompensation d'une cardiopathie remontant à la petite enfance.

Observation 2. M. Bez (I.B. 71 1453) 67 ans, est porteur depuis quatre ans d'une LLC stabilisée par administration continue de chlorambucil quand il est hospitalisé pour un tableau de décompensation avec fièvre et augmentation des adénopathies dans tous les territoires superficiels tandis que la lymphocytose reste stable autour de 20 000/mm³ avec 90% de petits lymphocytes au myélogramme. La lymphographie ayant révélé de très volumineuses adénopathies rétropéritonéales, on réalise d'abord une irradiation large de tous les territoires ganglionnaires sous-diaphragmatiques. La fonte de ces adénopathies est rapide mais on observe simultanément une progression des ganglions cervicaux et axillaires. La biopsie d'un ganglion cervical permet d'affirmer l'existence d'un réticulosarcome dictio-syncitial. Le malade reçoit alors une chimiothérapie associant vincristine, cyclophosphamide et prednisone. La régression tumorale atteint 70% mais est de brève durée et la reprise évolutive entraîne le décès du malade deux mois après l'apparition des signes de décompensation.

Observation 3. M. Fil (I.B. 71 1947) né en 1898, présente en avril 1971 un zona conduisant à la découverte d'adénopathies axillaires et cervicales modérées avec une lymphocytose sanguine à 15 900/mm³ et 80% de petits lymphocytes au myélogramme. Cette LLC paraît stable et n'est pas traitée. Un amaigrissement très important entraîne l'hospitalisation du malade début octobre 1971. Il présente une polyadénopathie diffuse et modérée avec une lymphocytose sanguine à 26 400. La biopsie d'un ganglion cervical permet d'affirmer un réticulosarcome dictio-syncitial. Une dysphagie conduit à découvrir une sténose œsophagienne due à un carcinome épidermoïde peu différencié qui est irradié par cyclocobalthérapie. Simultanément est administrée une association de vincristine, cyclophosphamide et prednisone entraînant une régression ganglionnaire de 75%. A l'issue de son irradiation, le malade rentre chez lui avec une chimiothérapie d'entretien mais la dysphagie qui a été peu améliorée s'accompagne d'une cachexie progressive responsable du décès du patient début janvier 1972 sans que se soit manifestée de reprise évolutive de son hémopathie.

Observation 4. M. Alp (I.B. 68 1256) 72 ans, présente en juillet 1968 une altération générale qui conduit à découvrir une polyadénopathie prédominant en régions inguinales, une lymphocytose sanguine à 54 600 et une infiltration massive de la moelle sternale par de petits lymphocytes. L'abstention thérapeutique initiale est interrompue en janvier 1970 en raison d'une ascension de la lymphocytose au-dessus de 100 000/mm³. Un traitement de six mois par chlorambucil rétablit une situation plus satisfaisante qui se maintiendra jusqu'en décembre 1971. A la fin de ce mois le malade est réhospitalisé avec une fièvre élevée, une atteinte marquée de l'état général, une croissance rapide de toutes les adénopathies, une hépatospléno

mégalie importante et une lymphocytose sanguine à 94 000. La biopsie d'un ganglion cervical permet de porter le diagnostic d'une forme réticulaire de la maladie de Hodgkin. La chimiothérapie associant vinblastine, procarbazine et cyclophosphamide entraîne une chute de la lymphocytose à 15 000 mais la fonte tumorale est moins nette et le maintien d'un traitement d'entretien par vinblastine n'empêche pas une reprise évolutive en avril 1972 rapidement fatale.

Commentaires

Ces quatre observations, étayées par une revue de la littérature, détaillée dans la thèse de VALRABOURG [2] amènent à distinguer, dans le cadre du syndrome de Richter, plusieurs types d'associations.

Dans un premier groupe d'observations les deux composantes du syndrome sont reconnues simultanément ou à quelques mois l'une de l'autre comme dans notre observation 3. Dans ce cas, il est impossible d'établir la chronologie exacte des deux perturbations. On ne peut exclure qu'il s'agisse d'une réaction leucémoïde lymphocytaire à une réticulopathie maligne, encore que ce type de réaction leucémoïde soit rare.

Pour les autres observations il existe un décalage important entre la reconnaissance initiale de la LLC et celle de la réticulopathie. (Les observations inverses de LLC survenant chez un malade suivi pour réticulopathie paraissent devoir être exclues du cadre du syndrome de Richter.) Dans ce cas plusieurs éventualités peuvent être schématiquement distinguées. Pour la plupart des cas, comme pour nos observations 2 et 4, la LLC persiste, inchangée, et l'on observe l'addition de la réticulopathie maligne. Il paraît logique alors d'assimiler cette situation à celle, plus générale et bien connue, de «LLC + cancer». Cette tendance est appuyée par les quelques observations où un autre cancer s'ajoute au syndrome de Richter, comme dans notre observation 3. Dans quelques observations au contraire, on constate, comme dans notre observation 1, une disparition spontanée des stigmates de la LLC au moment où se manifeste la réticulopathie donnant l'impression d'une substitution de la seconde maladie à la première et permettant seule d'évoquer une transformation de LLC en réticulopathie maligne d'évolution plus aiguë. Ces cas sont exceptionnels puisque nous n'en avons retrouvé que cinq autres dans la littérature. A côté de ces deux types bien tranchés se rangent toutes les observations où l'apparition de la réticulopathie maligne s'accompagne d'une réduction des signes de LLC et en particulier de la lymphocytose sanguine qui reste toutefois anormalement élevée. On ne peut actuelle-

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Tabelle 1 Halbquantitative Enzymaktivitätsbestimmung in Megakaryozyten der Ratte bei unterschiedlicher Dosierung von Cyclophosphamid

Dosis mg/kg KG	Reaktionsstufen														
	Laktathydrogenase					Alkalische Phosphatase					Saure Phosphatase				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1 × 10	0	1	11	8	5	0	8	9	5	3	9	7	7	2	0
1 × 25	0	5	17	3	0	5	15	5	0	0	4	12	9	0	0
1 × 50	0	3	15	6	1	13	10	2	0	0	1	5	14	4	1
1 × 75	1	6	10	6	2	8	11	6	0	0	1	6	13	5	0
1 × 100	0	10	11	4	0	9	10	6	0	0	0	5	16	4	0
Kontrollen	0	0	11	8	6	6	8	10	1	0	16	6	3	0	0
2 × 10	2	9	8	3	1	12	5	7	1	0	3	5	9	8	0
2 × 25	0	2	12	11	0	10	11	4	0	0	9	11	5	0	0
2 × 50	-	-		-	-	9	9	6	1	0	3	12	11	0	0
2 × 75	-	-	-	-	-	13	9	3	0	0	10	9	4	2	0
2 × 100	-	-	-	-	-	13	9	3	0	0	-		-	-	-

LDH, AP und SP waren in Megakaryozyten der Ratte zytochemisch nachweisbar. Aktivitätsveränderungen der drei Enzyme setzten synchron mit dem Thrombozytenabfall im peripheren Blut schon bei niedrigster Dosierung von Cyclophosphamid (10 mg/kg Körpergewicht) ein. Nur bei der LDH konnten wir mit steigender Dosierung eine Korrelation von Aktivitätsverlust und Thrombozytenzahl finden.

1. LDH. Nach einmaliger Injection von 10, 25, 50, 75 und 100 mg Cyclophosphamid/kg Körpergewicht (KG) sank die LDH-Aktivität linear leicht ab (Abb. 1, Tab. 1). Bei Dosierungen von 200, 250 und 500 mg/kg KG erfolgte der Abfall steil. Bei 500 mg/kg KG zeigte sich in den wenigen noch vorhandenen Megakaryozyten keine Aktivität mehr. Der Aktivitätsverlust war in Zellkern und Zytoplasma gleichmässig (Abb. 2). Bei zweimaliger Injektion von 10 und 25 mg/kg KG wurden noch mittlere Aktivitäten (bis Reaktionsstufe 3) beobachtet. Dagegen war nach zweimaliger Injektion von mehr als 50 mg/kg KG Aktivität nur noch in Spuren vorhanden. Die Abnahme des Enzymgehaltes unter Cyclophosphamid ist nach unserer Auffassung auf eine Synthesehemmung zurückzuführen, die sich ihrerseits durch RNS-Hemmung erklären lässt. Untersuchungen am Rattenhoden [5] führten zu vergleichbaren Ergebnissen.

2. AP und SP. Beide Enzyme verhielten sich in ihrer Dosis-Aktivitäts-Relation unterschiedlich. Bei der AP wurde unter geringen Cyclo-

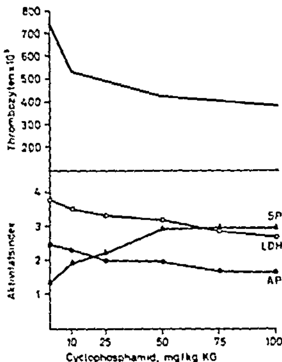


Abb 1. Verhalten der Enzyme LDH, AP und SP in Megakaryozyten sowie der Thrombozyten im peripheren Blut unter verschiedenen Cyclophosphamiddosen

tronics, Krefeld) gezahlt 26 Ratten wurde einmal, weiteren 26 zweimal im Abstand von 24 h Cyclophosphamid i.p. in unterschiedlicher Dosierung appliziert 24 h nach der letzten Injection wurde nach nochmaliger Thrombozytenzählung Knochenmark aus dem Femur punktiert Die Punktate wurden ausgestrichen, luftgetrocknet, in Aceton fixiert und histochemisch auf LDH nach PEARSE [7], AP nach WACHSTEIN [12] und SP nach GOMORI [3] untersucht 20 Tiere des gleichen Stammes blieben unbehandelt und dienten als Kontrollen

In Anlehnung an MITZKAT und ZURWEHME [6] wurden die Ausstrichpräparate halbquantitativ ausgewertet Jeweils 25 Megakaryozyten wurden ausgezählt und nach Intensität der histochemischen Reaktion 5 Aktivitätsstufen zugeordnet

Ergebnisse und Diskussion

Wie bereits von anderen Autoren beschrieben [1, 4], fanden wir nach Cyclophosphamid eine deutliche Depression aller Arten von Knochenmarkszellen Die Megakaryozytenzahl war um so niedriger, je höher die Cyclophosphamiddosis war Dieses Verhalten lässt sich durch Hemmung der DNS-Synthese erklären

Acta Haemat. 59: 223-227 (1973)

Some Aspects of Leucocyte Behaviour in Haemodialysis

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Abstract. Neutropenia early in haemodialysis is probably due to the return into the circulation of leucocytes damaged by the first contact with the dialyzing surface. The phenomenon finishes because the damaging action of the membranes may be rapidly eliminated by a protective film of haematic derivation. We did not observe any modifications of the leucocytes on re using (in the same dialysis) the same dialyzer, provided it is not rinsed. The leucocyte values immediately before the end of dialysis are lower than the starting values. This phenomenon is due to the progressive sedimentation of white cells on the dialyzing membranes during haemodialysis.

Key Words
Haemodialysis
Leucocytes in haemodialysis
Leucopenia

In 1968, KAPLOW and GOFFINET [3] described a profound transient neutropenia with fall of monocytes early in haemodialysis, the number of lymphocytes remaining the same. The phenomenon was ascribed to a transient sequestration *in vivo*, principally in the lungs. The leucopenic phase is followed by rapid recovery with rebound 1-3 h after the beginning of dialysis. The recovery is due to a re-appearance of sequestrated granulocytes and to a significant response of the bone marrow. The phenomenon is constant, independent on the type of kidney disease, on the number of dialysis performed, on the type of dialyzer used. It also appears in a healthy dialyzed dog. It is caused neither by heparin, nor by initial bleeding, nor by extracorporeal circulation (in fact, it does not appear by-passing the dialyzer). The phenomenon is induced neither by rapid infusion of 150-200 ml of dialyzing fluid which issues from the compartment of the dialyzer, nor by infusion of blood that has previously been in contact with the dialyzer. Neutropenia can be reproduced during the same dialysis by changing the dialyzer after the white count has returned to normal [1, 3-6].

[illegible]

[illegible]

[illegible]

[illegible]

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Abstract. Neutropenia early in haemodialysis is probably due to the return into the circulation of leucocytes damaged by the first contact with the dialyzing surface. The phenomenon finishes because the damaging action of the membranes may be rapidly eliminated by a protective film of haematic derivation. We did not observe any modifications of the leucocytes on re-using (in the same dialysis) the same dialyzer, provided it is not rinsed. The leucocyte values immediately before the end of dialysis are lower than the starting values. This phenomenon is due to the progressive sedimentation of white cells on the dialyzing membranes during haemodialysis.

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The purpose of the present paper is to try to clarify the dynamics of the phenomenon Moreover, following our observation [2] of a progressive leucocyte sedimentation on the dialyzing membranes during haemodialysis, we have studied the behaviour of leucocytes at the end of dialysis

Material and Methods

In 16 patients dialyzed twice a week (table I) the white cell count was done on microscopy before the start of dialysis 20 min after immediately before the end and 15 min after the end

In 5 of these patients the count was also performed before and after the change of dialyzer (at about the second hour of dialysis) On these same patients we also carried out the following experiments interruption of dialysis at the second hour, recovery of blood by air, immediate restarting of dialysis with the same dialyzer which we re used without washing on one occasion and on another occasion after washing with 1 000 ml of 0 9% sodium chloride The white count was performed before the interruption and during the phase after the reconnection of the patient

Finally we have studied the behaviour of leucocytes also in a healthy man submitted to a dialysis lasting 25 min (circulation vein to vein with pump blood flux in dialyzer 60–70 ml min)

Results

In the first phase of haemodialysis, a remarkable fall of granulocytes and monocytes appears in our uraemic patients, on the contrary, there is a low fall of lymphocytes The same kind of changes in the white cell count are seen in a healthy dialyzed man In patient No 13 (treated with vinblastine), the fall in the white count is much lower than in all other patients, it is practically non-existent in a second study performed when the starting neutrophil values are much more reduced The leucocyte values immediately before the end of dialysis are lower than the starting values, 15 min after blood recovery, we can see a constant and significant increase of leucocytes which reach the pre dialysis levels (table I)

We confirm that the phenomenon re-appears more than once during the same dialysis by changing the dialyzer after the white count has returned to normal Neutropenia, on the contrary, does not appear again if the patient is reconnected to the same dialyzer after blood recovery The phenomenon manifests itself in such a situation if the dialyzer is rinsed before re using (fig 1)

Table 1 Haemodialysis

Case No	Age years	Sex	Months of dialysis	Diagnosis	Leucocytes/mm³ (granulocytes/mm³) I	II	III	IV
1	41	M	33	chronic pyelonephritis	5 500 (4 000)	1 100 (180)	5 000 (3 500)	6 800 (4 600)
2	42	M	22	chronic nephritis	3 400 (2,700)	1 000 (200)	2,000 (1,500)	3 300 (2,600)
3	33	M	3	chronic nephritis	6,100 (4 600)	1,200 (300)	4 900 (3 500)	5 700 (4,200)
4	43	M	8	chronic nephritis	5 600 (3 350)	1 100 (350)	5 000 (3 000)	6,800 (4,200)
5	42	M	16	chronic nephritis	5,200 (4 100)	900 (200)	3 700 (2,800)	5 000 (4 000)
6	45	M	12	chronic nephritis	4 400 (3 100)	1 000 (260)	3 000 (2 000)	4 100 (3 200)
7	32	M	10	chronic nephritis	5 000 (3 400)	1 100 (300)	2,800 (1 700)	5 000 (3 300)
8	40	F	16	polycystic kidney	5 800 (4 900)	700 (160)	4 300 (3,200)	5 800 (4 600)
9	19	F	44	chronic nephritis	6 000 (4 800)	1 000 (100)	4,300 (3,200)	4 300 (3 300)
10	11	M	20	chronic nephritis	7 000 (5 700)	1,200 (210)	4 000 (3 400)	6,500 (5 200)
11	30	M	16	chronic nephritis	4 100 (3,200)	2,800 (1,200)	3 800 (3 000)	4 400 (3,300)
12	44	M	5	chronic nephritis	5 100 (3 950)	1 400 (300)	4 000 (3 300)	4 900 (3 800)
13	24	M	18	cortical necrosis Hodgkin	2,200 (1 500)	1 000 (700)	1 600 (1 000)	3 600 (2,400)
13a	second study				1 200 (1 000)	900 (700)	–	–
14	42	M	33	amyloid kidney	7 000 (5 000)	1 000 (100)	3 000 (1 950)	5 800 (3 900)
15	57	F	0 5	chronic nephritis	8 100 (6 600)	1 700 (400)	6,100 (4 800)	8 000 (6,500)
16	39	M	–	healthy	8 400 (5 900)	3 300 (1,200)	–	–

Twice a week, patients undergo haemodialysis lasting from 11 to 12 h with Kiil kidney. White count is done on microscopy before the start of dialysis (I), 20 min after (II), immediately before the end (III), and 15 min after the end (IV). Before and after the end we have not observed significant modifications in the differential white count. In the healthy man we see the usual fall in neutrophils early in haemodialysis. *Granulocyte* values in parentheses.

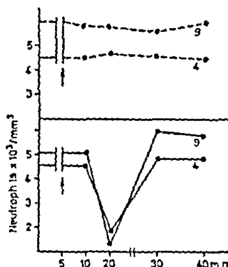


Fig. 1. The re-use of the same dialyzer late in haemodialysis (recovery of blood by air without washing) does not induce any leucocyte modifications (upper panel). The neutrophils fall if the dialyzer is washed before re-use (lower panel). Experiments were performed at the second hour in 2 different haemodialyses. The arrows indicate the time of reconnection of the patient to the dialyzer (9 and 4 = case No., see table I).

Discussion

Neutropenia early in haemodialysis, due to a reversible sequestration *in vivo*, appears after the flux of a critical amount of blood through the dialyzer. The phenomenon ends spontaneously. It re-appears during the same dialysis when the patient is connected to a fresh dialyzer. The re-use of the same dialyzer (late in dialysis) does not cause neutropenia, it appears, on the contrary, if the dialyzer is washed before re-use.

We can formulate a work hypothesis which explains the different aspects of the phenomenon – defined 'a fascinating puzzle' by SMITH and JOBBINS [5]. The sequestration *in vivo* (which induces neutropenia) could be a self multiplying phenomenon caused by the return to the patient of an amount of leucocytes which have been modified by the first contact with the membranes – we recall the experimental leucopenia by transfusion of alterated leucocytes [7]. The process finishes because the damaging power of the dialyzing surface comes rapidly to an end. The demonstration that the re-use of the same dialyzer does not cause leucocyte modifications, provided the chambers are not rinsed, suggests that a pro-

tective film from the blood eliminates the damaging properties of the membranes a simple washing can alter this layer

The second aspect of leucocyte behaviour in haemodialysis focused by us is the final decrease our data agrees with those published by PAPADIMITRIOU *et al* [4]

We think that this phenomenon depends on the sequestration of leucocytes in the dialyzer during haemodialysis [2]

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Acta Haemat 50 228–237 (1973)

Congenital Hypoproconvertinemia (Factor VII Deficiency)

A Report of Two Cases Belonging to Two Different Kindreds

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Key Words
Bleeding disorders
Blood coagulation
Factor VII deficiency
Hypoproconvertinemia

Abstract Two new cases of congenital factor VII deficiency are reported They are two 7 year-old boys belonging to two different kindreds The main clinical features were bleeding from the gums, epitaxis (one patient) easy bruising melena, post-traumatic and spontaneous hemarthrosis The main coagulation features were prolonged prothrombin time, normal Stypven-cephalin clotting time, normal first stage coagulation tests, normal platelet and vascular tests. The prothrombin time was corrected by the addition of normal serum factor II or factor V or factor X-deficient plasmas and of the abnormal factor X (factor X Friuli) plasma On the contrary, the addition of ad sorbed normal plasma and of three plasmas with known factor VII deficiency failed to correct the abnormality The factor VII levels were found to be 2.3 and 2.7% of normal, respectively The parents of the patients were not consanguineous But 3 of them, together with other members of the 2 families, were found to have slightly decreased factor VII levels and were considered to be heterozygotes for the defect. These findings are consistent with an incompletely recessive pattern of autosomal inheritance

Congenital factor VII deficiency is still considered to be a rare coagulation disorder. So far, only about 56 sufficiently proved cases have been reported in the world literature 39 'proved' or 'probable' cases were gathered by OWEN *et al* in 1964 [21] We found 11 additional cases in a reperusal of the papers appearing before or during 1964 [2, 7, 8, 13, 19] Since 1965, only 6 cases have been reported [6, 12, 18, 20] Several 'probable' cases of factor VII defect have been described, but final proof of the nature of the defect in these cases is lacking [21] This

is particularly true for the cases reported before 1956 or 1957, the years in which factor X defect was first recognized. The matter is further complicated by the recent finding that one of the criteria commonly maintained to indicate a factor VII defect (a normal Russell's viper venom clotting time together with a prolonged prothrombin time) is no longer true [4]. In fact, in the abnormal factor X (factor X Friuli) coagulation disorder, the Stypven-cephalin clotting time is normal whereas the prothrombin time is prolonged and the factor VII level is normal. The peculiar behavior is due to the fact that the abnormal factor X can be activated normally by Russell's viper venom [4].

The object of this paper is to report on 2 patients with sure factor VII congenital defects together with family studies. These are the fifth and the sixth patients with this defect reported in Italy. The previous patients were reported recently by others and by us [6, 20].

Materials and Methods

Material and method used were reported in detail elsewhere [3, 4]. Only new data will be given herein.

Three factor VII-deficient plasmas were used in cross experiments and in factor VII assay. The first plasma was kindly supplied in lyophilized form by Dr. C. Owen, Department of Pathology, Mayo Clinic, Rochester, Minn., USA. Another lyophilized factor VII-deficient plasma was supplied by Dade Laboratories, Miami, Fla., USA. We also used the noncontacted frozen plasma of another patient with congenital hypoproconvertinemia [6].

All factor VII assays were carried out using as substrate equal parts of lyophilized Dade factor VII-deficient plasma and adsorbed normal plasma. All the test plasmas assayed were nonconcentrated plasmas but the assay was carried out in normal glassware.

Case I, a 7-year-old boy, was first seen by us in 1972.

The family history was negative for bleeding disorders, and the parents were not consanguineous (fig. 1). The patient was born at term, and no undue bleeding was noted in the neonatal period. With the appearance of the first teeth, ecchymosis of the gums was noted.

At the age of 1½ years, the patient underwent minor surgery for the correction of a phimosis. Only moderate bleeding was noted.

At 2 years, the patient started complaining of frequent epistaxis and had to be admitted several times to a local hospital.

At 3 years, the patient presented a large posttraumatic hematoma of the right flank and had to be admitted to another hospital. Several whole blood transfusions were given, and the hematoma slowly subsided.

■ = propositus homozygote, symptomatic;
◧ or ◐ = heterozygotes asymptomatic;
□ or ○ = studied asymptomatic, normal;
□ or ○ = not studied asymptomatic, normal?

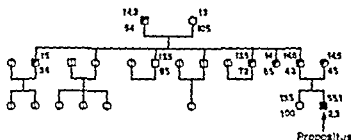


Fig 1 Family pedigree of case 1. The upper numbers on the right or left side of each square (male) or circle (female) refer to the prothrombin time in sec, the lower numbers refer to the factor VII percentile level

■ = propositus homozygote symptomatic;
◧ or ◐ = heterozygotes asymptomatic;
□ or ○ = studied, asymptomatic normal;
□ or ○ = not studied asymptomatic normal?;
■ = deceased, asymptomatic, normal?

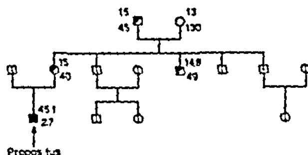


Fig 2 Family pedigree of case 2. The numbers have the same meaning as in figure 1

During the past 3 years the patient has suffered several bleeding episodes: melena, hematuria, posttraumatic hemarthrosis of both elbows, and a posttraumatic hemarthrosis of the left knee. On every occasion the patient had to be hospitalized and was treated with whole blood or plasma transfusions. No functional limitations of the joints remained after the hemarthrosis. During the last admission a tentative diagnosis of factor VII deficiency was formulated, and the patient was sent to us for further studies

Case 2 a 7 year-old boy, was also first seen by us in 1972.

The patient was born at term to an unwed mother, and no bleeding disorder was noted at birth. Family history on the maternal side was negative for bleeding disorder. The father was not available for information but no bleeding tendency seemed to be present in his family either (fig. 2)

The patient started complaining of bleeding from the gums at the age of 2½ years. At 3 years the patient started to bruise easily. Epistaxis on the other hand, was rare. At 5 years, the patient bled severely from the gums, experienced mild hematuria, and had to be admitted to a local hospital. The patient was transfused with whole blood or plasma, and bleeding subsided promptly.

Since the age of 5 years, the patient has presented several episodes of hemarthrosis in practically all major joints, but particularly in the left elbow. These hemarthroses usually followed traumata of variable intensity; occasionally no trauma history could be elicited and the episodes were considered spontaneous. The repeated hemarthrosis of the left elbow led to a mild to moderate ankylosis of the joint.

At 6 years the patient presented another episode of gingival bleeding which required another hospital admission. During the past year he presented hemarthrosis of both ankles and 2 episodes of posttraumatic bleeding from the gums. In all instances the patient had to be hospitalized and was treated with whole blood or plasma transfusions.

No bleeding manifestations were presented at the time of study. Physical examination revealed the presence of a slight underweight and a limited function of the left elbow joint. The patient was able to extend the arm only about 160°.

Results

The results of the coagulation studies are summarized in tables I–III. The prothrombin time was prolonged and corrected by addition of normal plasma, though not by addition of adsorbed normal plasma. Plasma congenitally deficient in factor II or V or X and plasma with the abnormal factor X (factor X Friuli) disorder corrected the abnormality. Yet 3 plasmas with known factor VII deficiency failed to correct it. The prothrombin time of plasma mixture was also prolonged in the 2 cases. Glass clotting time, recalcification time and the thromboelastogram were perfectly normal. Partial thromboplastin time, prothrombin consumption and the thromboplastin screening test were normal too. The thromboplastin generation test was normal or showed a very mild serum defect (fig 3). Platelet tests and bleeding time were within normal limits, and there was no hyperfibrinolysis.

The factor VII level found in the plasma of our cases was 2.3 and 2.7% of normal, respectively. Both parents of case 1 and the mother of

Table I Complete coagulation studies in case 1. Similar results were obtained in case 2

	Case 1	Normal values
Platelet count	250 000	150 000–350 000
Bleeding time, min	3½	2–5
Clot retraction	complete after 6 h	complete after 12 h
Glass clotting time, min	8½	5–10
Partial thromboplastin time, sec	40.5	35–45
Prothrombin consumption, sec	>90	>90
Thromboplastin screening test	16 sec in 8 min	<15 sec in 6 or 8 min
Thromboplastin generation test	18 sec in 8 min	<16 sec in 6 or 8 min
PT, sec	55.1	13–14
PP test (Owen), sec	91.6	26.6
Stypven-cephalin clotting time, sec	12	11–13
Thrombin time, sec	18	18–25
Fibrinogen, mg%	310	250–450
Factor II (one stage), %	98	85–115
Factor II (two-stage), %	95	85–115
Factor V, %	100	85–115
Factor VII, %	2.3	85–115
Factor VIII, %	110	70–150
Factor IX, %	100	80–130
Factor X, %	100	85–115
Factor XI, %	120	60–150
Factor XII, %	110	60–150
Thromboelastogram		
r, mm	17	10–20
K, mm	6	6–12
ma, mm	62	50–66

case 2 together with other family members had slightly decreased factor VII values and were considered to be heterozygous for the abnormality. These heterozygotes were all asymptomatic (fig. 1, 2).

Discussion

The main criteria for diagnosis of factor VII deficiency are: (1) prolonged prothrombin time corrected by addition of normal serum, (2) normal or near normal first-stage tests, and (3) normal Stypven-cephalin clotting time. Our patients meet these criteria fully. The lack of correc-

Table II. Prothrombin time correction studies in case 1. Similar results were obtained from the other patient

Mixture, equal parts	Clotting time sec	Reference plasma PT sec	Comment
Plasma	55.1		
Plasma + adsorbed normal plasma	46.0		
Plasma + normal serum	17.3		
Plasma + factor VII-deficient plasma	54.0	45.0	Lyophilized factor VII-deficient plasma received from Dr. Owen
Plasma + factor VII-deficient plasma	44.5	40.1	Dade lyophilized factor VII-deficient plasma
Plasma + plasma of another patient with factor VII deficiency	53.0	48.5	Noncontacted frozen plasma, personal case
Plasma + factor X-deficient plasma	16.5	72.5	Dade lyophilized factor X-deficient plasma
Plasma + abnormal factor X coagulation disorder (factor X Friuli)	16.2	33.3	Frozen plasma, personal case
Plasma + factor II-deficient plasma	16.0	21.0	Frozen plasma, personal case
Plasma + factor V-deficient plasma	15.8	40.0	Frozen plasma, personal case

tion of the prothrombin time after addition of the plasmas of 3 known cases of factor VII deficiency establishes the diagnosis unequivocally. The slight abnormality observed in the thromboplastin generation system found in one of our cases is not surprising since this has already been seen in other proved factor VII deficient cases [6, 21]. This could indicate that factor VII plays a minor role even in the thromboplastin generation. The studies which deal with the relationship between the

Table III Main laboratory findings in case 2. Unreported findings were within normal limits

	Case 2	Normal values	Comment
PT, sec	45 1	13	
Factor VII *,	2.7	85–115	
Partial thromboplastin time, sec	39	35–45	
Stypven-cephalin clotting time sec	13	11–13	
PT mixture patient plasma + case 1 plasma, sec	48		Case 1 plasma was non-contacted frozen plasma
Thromboplastin generation test	16 sec in 8 min	<16 sec in 6 or 8 min	

blood-coagulation contact-phase system and factor VII seem to support this contention [16]

There are no typical bleeding manifestations in patients with factor VII deficiency The severity of bleeding is variable Epistaxis, bleeding from the gums, easy bruising and menorrhagias are the commonest bleeding manifestations [21] Hemarthroses are common but are often posttraumatic The frequency of hemarthrosis seems to be definitely higher than in other prothrombin complex factor deficiencies

OWEN *et al* [21] have postulated that 2 types of factor VII deficiency exist on the basis of an early or late onset of bleeding symptoms However, this assumption is incorrect, since it was based on the data concerning 2 patients who were thought to have factor VII deficiency They were subsequently found to have the abnormal factor X (factor X Friuli) coagulation disorder [4]

The occurrence of bleeding in the patients with congenital factor VII deficiency is puzzling since the vascular, platelet and intrinsic coagulation systems are normal The coagulation defect consists only in an abnormally slow activation of factor X by the tissue thromboplastin + factor VII complex The most likely explanation is to admit that a good hemostasis *in vivo* is dependent on the presence of adequate proconvertin level Whether this is due to the fact that tissue thromboplastin plays an important role *in vivo* or due to some other unidentified mechanism remains to be proven

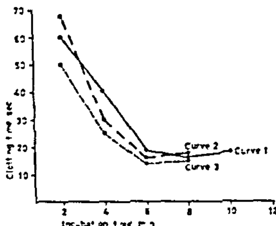


Fig 3 Thromboplastin generation test in case 2. The pattern is normal. Curve 1 is the basal curve. Curve 2 was obtained after the substitution of patient's adsorbed plasma with adsorbed normal plasma. Curve 3 was obtained after the substitution of patient's serum with normal serum. In case 1 a mild serum defect was noted.

Consanguinity was not present among the parents of our patients. This is not unusual since it was already seen in other cases [6, 21]. The pattern of inheritance seems to be incompletely recessive in autosomal type [6, 17, 23]. This assumption is fully confirmed by the present findings. The parents of case 1 and the mother of case 2 as well as other members of the family had decreased factor VII levels and were therefore considered heterozygotes. The father of case 2 is probably a heterozygote too. If he is not, a mutation has to be assumed to explain the homozygosity found in his son. Heterozygotes may be easily differentiated from the normal population on the basis of a specific factor VII assay.

However, our heterozygotes also almost always showed a 1- to 2-sec prolongation of the prothrombin time. In this regard it is interesting to note that heterozygotes for factor VII deficiency seem to be completely asymptomatic. We have seen 12 heterozygotes for this defect (9 related to the patient and 3 belonging to another family) [6]. None of them has noticed undue bleeding, even after surgical procedures, parturition, or traumata. This aspect is rather strange – and puzzling too, since heterozygotes have been reported to bleed unduly on occasion following minor

surgery or trauma [3–5, 10] related to other prothrombin complex factor deficiencies or abnormalities such as congenital hypoprothrombinemia, factor X deficiency, and the factor X Friuli coagulation disorder. Since the lowest factor VII levels found in these heterozygotes was about 34–45% of normal, it could be speculated that such levels are hemostatically sufficient.

Finally it has been shown recently that some factor VII-deficient plasmas contain anti-factor VII-reacting material [9]. These observations, if confirmed, would indicate the existence of genetic variants for the factor VII defect. Data concerning the genetic variants of the other prothrombin complex coagulation disorders have already been firmly established [4, 14, 16].

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Acta Haemat 50 238-244 (1973)

Platelet Defect in a Case of Ehlers-Danlos Syndrome

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Abstract In a case of Ehlers Danlos syndrome, a platelet defect is described It is characterized by a defect of ADP and platelet factor 3 release, absence of secondary curve in ADP, adrenalin and distilled water induced aggregation and a defective collagen aggregation It is pointed out that this defect, which occurs in primary thrombopathy, also exists in connective tissue diseases such as Ehlers Danlos syndrome, osteogenesis imperfecta, etc

Key Words
ADP of platelets
Connective tissue diseases
Ehlers Danlos syndrome
Platelet aggregation
Platelet factor 3
Thrombopathy

The Ehlers-Danlos syndrome is a rare hereditary connective tissue disease characterized by hypermobility of joints, hyperelasticity of skin, easy bruising and hemorrhagic tendency [5, 9, 18] It is genetically determined and transmitted by the autosomal dominant mode of inheritance Bruising tendency is frequently present and orthopedic, gastrointestinal, cardiovascular and ocular disturbances may occur Other features which are sometimes found are calcified subcutaneous spheroids and molluscoid pseudotumors

Although the presence of hemorrhagic tendency in this syndrome has long been observed, our knowledge is still limited regarding its pathogenesis [24] LISKER *et al* [16] have reported factor IX deficiency in 2 cases of Ehlers Danlos syndrome On the other hand, platelet defects have also been reported by GOODMAN *et al* [8], KASHIWAGI *et al* [13] and ESTES [7]

In this paper, a case of Ehlers Danlos syndrome associated with a platelet defect is presented

[1] Haemostasis Research Unit supported by the Scientific and Technical Research Council of Turkey

Methods

Platelets were counted according to the method of BRECHER and CRONKITE [3]. Bleeding time was performed by the method of IVY *et al.* [12]. Coagulation time was determined by the method of LEE and WHITE [15]. One-stage prothrombin time was measured by the method of QUICK [20] and two-stage prothrombin time was determined by the method of WARE and SEEGERS [32]. Fibrinogen determination was performed according to RATNOFF and MENZIE [21]. Partial thromboplastin time was determined by the method of LANGDELL *et al.* [14]. Platelet factor 3 availability was performed by the method of SPAET and CINTRON [23]. Platelet factor 3 activity was determined using modified thromboplastin generation test [2, 30] (the platelet counts in the final solutions were adjusted to 100 000/mm³). Euglobulin lysis time was performed as described by COPLEY *et al.* [6]. Factor VIII assay was determined by the method of PITNEY [19] and factor IX with the method of BARROW and GRAHAM [1]. Factor XIII determination was done using the method of LORAND and DICKENMAN [17] and SIGG's method [22].

Platelet aggregation studies were done using Bryston aggregometer (Bryston Manufacturing Ltd, Rexdale, Ont., Canada) and Bausch and Lamb recorder. For aggregation curves in the final solution, ADP was 0.5 µg/ml and adrenalin was 25 µg/ml. Distilled water induced aggregation was performed as described by ULUTIN and ULUTIN [31]. Collagen was obtained and prepared from the human rectus abdominus fascia.

ADP determination of platelet was done by using Boehringer enzyme sets and read at 366 mm (UV) with Beckman DU 2 spectrophotometer.

Case Report

The patient is a 47 year-old male. He was complaining of pain in both hips, elbows and dorsal region. He had been a full term baby and his birth had been normal. Neither his parents nor any of their known relatives had any stigmata of the Ehlers-Danlos syndrome. He was complaining ever since his childhood of easy bruising and ecchymoses after slight trauma. He suffered from a hypermobility of joints in his childhood. He had limitation of motion secondary to frequent sprains and strains followed by blood effusion and swelling. A gastrointestinal bleeding occurred when he was 43 years old.

Mild kyphoscoliosis. Limitation of flexion and extension of lumbar spine. Loss of physiologic lumbar lordosis. There was an old rupture at the myotendinous junction of the right vastus medialis muscle. Several subcutaneous painless nodules of different size were present at the dorsal aspect of elbow bilaterally. There was also limitation of flexion and extension at both elbow joints.

Electromyography (with conduction velocity of radial and fibular nerve conduction) was found to be within normal limits.

X-ray examination revealed calcified subcutaneous nodules in the region of great trochanter and olecranon bursa bilaterally. Heads of radii were dislocated and

Table I Laboratory findings

Bleeding time, min	8
Coagulation time, min	5–6
Retraction	normal
Platelet count	200 000/mm³
Prothrombin time, sec	15 (normal 15)
Two-stage prothrombin, U/ml	200
Fibrinogen, mg%	294
PTT, sec	64 (normal 60)
Platelet factor 3 availability	decreased
Platelet TGT	decreased
After frozen and thawed	normal
Euglobulin lysis time, min	160
F VIII, %	80
F IX, %	110
F XIII	normal

Table II ADP values in mg/3 × 10¹⁰ platelets are measured after incubation with collagen

	Supernatant PPP/mg	Platelets	Release of ADP, %
Patient	0.525	1.225	30
Control	1.470	0.720	67

there was an irregulability of joint surfaces and narrowing of the joint spaces. Examination of the spine revealed kyphoscoliosis and a marked osteoporosis.

Routine laboratory findings were within normal limits.

Laboratory investigations regarding bleeding tendency revealed a qualitative defect of platelets as summarized in table I. Bleeding time was slightly prolonged. Platelet factor 3 activity and availability were decreased. ADP (fig. 1) and distilled water induced primary aggregation normal (fig. 2) but there were no secondary aggregation curves. Collagen induced aggregation was slightly defective (fig. 3).

Total ADP content of platelets was normal but collagen induced ADP release decreased significantly (table II).

Discussion

A platelet defect exists in our case where the secondary aggregation related to normal release phenomenon does not occur and where the colla-

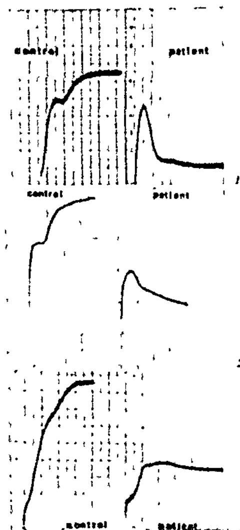


Fig. 1. Platelet aggregation induced by 0.5 µg ADP/ml
Fig. 2. Platelet aggregation induced by distilled water
Fig. 3. Platelet aggregation induced by collagen

gen induced aggregation is defective. Collagen induced ADP and platelet factor 3 release were also defective. The platelet defect of this case is similar to the findings in primary thrombopathy [25, 26]. In other words, in this case, an Ehlers-Danlos syndrome and primary thrombopathy exist together.

Although the existence of bleeding diathesis in Ehlers-Danlos disease is a known fact, there is very little literature dealing with the platelet defect in this kind of cases. GOODMAN *et al.* [8] observed in a family with Ehlers-Danlos syndrome the prolongation of bleeding time, the defect in prothrombin consumption related to platelet factor 3 deficiency and the existence of defective clot retraction. In a case of Ehlers-Danlos syndrome, KASHIWAGI *et al.* [13] found similar results with platelet factor 3 release defect and ultrastructural findings similar to primary thrombopathy.

In our case, we have found in the platelets a defective release of ADP and factor 3, an absence of secondary curve with ADP, adrenalin and distilled water and a defective curve with collagen which are similar to the findings in cases of primary thrombopathy [25, 26].

The primary thrombopathy is characterized by a defect in the platelet release mechanism, described in 1956 by …UTIN and KARACA [29, 30]. The authors drew attention to the fact … in some cases of primary thrombopathy several other congenital … could exist togethe [27]

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Acta Haemat. 50: 245–248 (1973)

Neuartige Einschlüsse im Ergastoplasma peripherer Lymphozyten bei Virusinfekt[1]

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Abstract. Novel inclusions were seen in the perinuclear space and in the cisternae of the rough surfaced endoplasmic reticulum in a lymphocyte of a patient suffering from rubeola.

Key Words
Electron microscopy
Endoplasmic reticulum
Lymphocyte structure
Rubeola

Etwa 3% peripherer Lymphozyten gesunder Personen enthalten ein ausgeprägtes Ergastoplasma (rough surfaced endoplasmic reticulum) [4]. Bei Virusinfekten (Rubeolen, Morbilli) nimmt die Zahl derartiger Ergastoplasma-reicher Zellen zu. Parallel hierzu steigt der Prozentsatz von Lymphozyten, in welchen fluoreszenzmikroskopisch IgG nachzuweisen ist [1]. Bei einer Patientin, die an Rubeolen erkrankt war, fielen uns im Ergastoplasma eines Lymphozyten Einschlüsse auf, welche in dieser Form noch nicht beschrieben wurden. Obwohl Entstehungsart und Bedeutung derartiger Strukturen unbekannt sind, sollen die Befunde kurz mitgeteilt werden, um die Variationsbreite morphologischer Zellreaktionen zu demonstrieren und einer Verwechslung mit spezifischen Veränderungen maligner Zellen vorzubeugen.

[1] Studie im Rahmen der Assoziation Hämatologie EURATOM-GSF, Nr. 031-64-1 BIAD.

[2] Wir danken Frl. M. [illegible] und Frl. G. [illegible] für die Mitarbeit.



1



2

Material und Methode

100 periphere Lymphozyten einer 24jährigen Rubeolenpatientin (Exanthemausbruch 3 Tage zuvor) wurden elektronenmikroskopisch ausgewertet [4]. Die Rötelnerkrankung nahm einen normalen klinischen Verlauf, insbesondere ergab sich kein Hinweis für eine maligne Erkrankung des lymphatischen Systems. Gleichzeitig wurden hier sowie bei anderen Patienten mit verschiedenen Virusinfekten immunzytologische, autoradiographische und elektronenmikroskopische Untersuchungen durchgeführt, deren Methodik und Ergebnisse mitgeteilt wurden [1].

Befunde

5% der Lymphozyten der Patientin zeigten ein deutlich vermehrtes Ergastoplasma. Die Ergastoplasmazisternen und der perinukleäre Spalt eines Lymphozyten waren erweitert und enthielten lamelläre Strukturen (Abb. 1, 2). Diese bestanden aus elektronendichten, weniger als 100 Å dicken Lamellen, die in einer Periodik von etwa 150 Å aneinandergelagert die Ergastoplasmazisternen sowie den perinukleären Spalt ausfüllten. Die Golgi-Zisternen waren frei von diesen Strukturen.

Diskussion

Den von uns beobachteten Strukturen entsprechende Einlagerungen wurden unseres Wissens bisher nicht beobachtet. Sie unterscheiden sich eindeutig von tubulären Zytoplasmaeinschlüssen normaler Lymphozyten [5] sowie von kristalloiden Granula bei lymphoretikulären Systemerkrankungen [2]. Langerhans-Granula bei malignen Histiozytosen [6, 8] sowie ähnliche Einschlüsse bei leukämischer Retikuloendotheliose [9] sind ebenfalls nicht mit ihnen zu verwechseln. Verdoppelungen der Ergastoplasmalamellen, die entfernt den von uns beobachteten Strukturen ähneln, wurden bei lymphoretikulären Systemerkrankungen mitgeteilt [3, 7].

Abb. 1. Lymphozyt mit lamellären Einschlüssen im perinukleären Spalt und Ergastoplasma [illegible]. Im Kern [illegible]. M = Mitochondrien. G = Golgi [illegible] × 11000

Abb. 2. Ausschnitt aus Abb. 1 [illegible]. Links der Kern angeschnitten (N). Im perinukleären Spalt und in den Ergastoplasmazisternen Lamellen [illegible]. Die Dicke der einzelnen Lamellen ist weniger als 100 Å. Abstand von Lamellenzentrum zu Lamellenzentrum etwa 150 Å. × 55000

Die Bedeutung der bei unserer Rötelnpatientin beobachteten lamellären, im Ergastoplasma und perinukleären Spalt lokalisierten Einschlüsse ist nicht bekannt. Sie demonstrieren die Variationsfähigkeit nichtmaligner Lymphozyten und machen deutlich, dass morphologische Befunde bei malignen Erkrankungen vorsichtig zu interpretieren sind.

Zusammenfassung

In den Ergastoplasmazisternen und im perinukleären Spalt eines Lymphozyten einer Rötelnpatientin wurden neuartige lamelläre Einschlüsse beobachtet.

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Sonderdruckbestellungen an: PD Dr. D. Huhn, Institut für Hämatologie, Landwehrstrasse 61, *D-8 München 2* (BRD)

Acta Haemat. 50: 249-256 (1973)

Pyknocytosis in Heat-Stroke

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Abstract. Large numbers of pyknocytes were observed in the peripheral blood of a Negro gold miner shortly after he developed heat stroke. Examination of these cells under the scanning electron microscope showed aggregation of the cell content to one pole and fragmentation of the surface membrane. Heat stroke was complicated by uraemia, acidosis and mild hepatocellular damage. Attempts to induce this morphological change *in vitro* by heating erythrocytes for varying times, at differing pHs and in uraemic serum were unsuccessful. The mechanism of production of pyknocytes in this patient is not known.

Key Words
Erythrocytes, heating in vitro
Heat stroke
Pyknocytosis
Scanning electron microscope

The effect of heat on erythrocytes has been studied both *in vitro* and, to a lesser extent, *in vivo*. At temperatures in the biological range of fever, increased osmotic and mechanical fragility occurs, with spontaneous haemolysis and shortened red cell survival [1, 4, 9, 10]. At temperatures greater than 47 °C, erythrocytes show in addition striking and irreversible morphological changes, especially the formation of spherocytes and microspherocytes [6]. Similar changes have been demonstrated in patients with thermal burns [4]. Perhaps the clinical condition in which the adverse effects of heat on man can best be assessed is heat stroke. Although the changes in certain organs have been documented, little is known about the effect of heat stroke on erythrocytes. We report here a patient in whom pyknocytosis and haemolysis were demonstrated following such an episode, and describe our attempts to reproduce this morphological

change *in vitro* The opportunity was taken to examine pyknocytes under the scanning electron microscope and the appearances are described

Case Report Including Special Investigations

M N a 25 year-old Negro from Malawi had been completely well before starting work on the gold mines He was fully acclimatised and had shown no evidence of heat intolerance during the 10 months he had been employed On 11th December 1971 after working for 4 h at a dry bulb temperature of 33 5 °C (92 3 °F) wet bulb temperature of 31 0 °C (87 8 F) with a wind velocity of 0 45 mps and a wet Kata reading of 9 6 units, he was seen to be confused mumbling and behaving inappropriately His skin was hot and dry and his eyes were staring Although his temperature was not taken a provisional diagnosis of heat stroke was made and cooling was commenced using a mixture of cold water and compressed air He became violent during the procedure and then lapsed into coma Cooling was continued for 1 h He was then brought to the surface and admitted to the mine hospital

On arrival the patient s blood pressure was 140 50 mm Hg pulse 160 beats per minute and rectal temperature 42 2 °C (108 0 °F) The skin was dry but there was no obvious dehydration The patient was comatose and swaying movements of the eyes were present Intermittent convulsions occurred for the first 24 h The heart and lungs were normal and the abdomen was slightly distended The urine contained protein cells and casts The blood urea level was 65 mg 100 ml serum sodium 140 mEq/l potassium 2 6 mEq/l chlorides 116 mEq/l carbon dioxide content 13 6 mEq/l and blood sugar concentration 135 mg/100 ml The serum glutamic oxaloacetic transaminase was 260 Reitman Frankel units glutamic pyruvic transaminase 130 Reitman Frankel units and lactic dehydrogenase (LDH) 330 Wroblewski La Due units Cooling was commenced and after 2.5 h the rectal temperature had fallen to 36 C (96 8 F) The patient was given 10 mg of diazepam and 40 mg of chlorpromazine by intramuscular injection and 500 mg of hydrocortisone intravenously Intravenous fluids were administered On the third day of illness the patient was transferred to the Johannesburg General Hospital for further management At this time the rectal temperature was 39 C (102 2 °F) He was semi comatose and had pin point pupils. There was no other obvious neurological deficit and the remainder of the examinatoin was unremarkable

The haemoglobin level was 14.5 g 100 ml haematocrit 47% erythrocyte count 4 6 million mm³ and platelets 40 000 mm³ The leucocyte count was 11 800 mm³ with a differential count of 82% neutrophils 2% monocytes and 16% lymphocytes Examination of a blood smear showed severe anisopoikilocytosis with numerous pyknocytes (45%) (fig 1) Coagulation studies revealed a prothrombin time of 12.5 sec a normal kaolin partial thromboplastin time fibrinogen 315 mg 100 ml factor VIII assay 142% and factor V assay 110% Fibrinogen degradation products were slightly increased at a level of 25 6 μg ml (normal 4 10 μg ml) The blood urea level was 130 mg 100 ml and the serum electrolytes were normal The blood urea concentration remained at approximately the same level for 5 days before rising sharply to 319 mg 100 ml The serum bilirubin concentration was

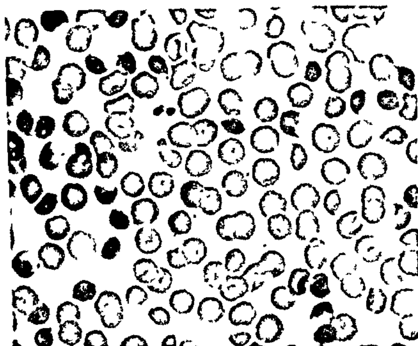


Fig. 1. Peripheral blood smear showing the presence of many pyknocytes.

4.9 mg/100 ml on admission, reached a maximum of 6.6 mg/100 ml the following day, and decreased thereafter reaching 2.4 mg/100 ml on the tenth day.

Both urobilinogen and bilirubin were present in the urine during the first day. Subsequent haematological studies showed little alteration in the haemoglobin level but the leucocyte count rose to 17,600/mm³. Reticulocyte levels were mildly increased, rising to 8.0% 6 days after admission. Thrombocytopenia persisted at the original level. Plasma haemoglobin levels were raised at 20.8 mg/100 ml (normal 2–[illegible] mg/100 ml) in association with persistent haemoglobinuria. The number of pyknocytes decreased progressively and when blood was taken for the last time on the tenth day constituted 1[illegible]% of the peripheral smear. Bone marrow aspiration on the eighth day showed a normocellular marrow. Erythropoiesis was normoblastic and of slightly decreased activity. Granulopoiesis was normal. The myeloid: erythroid ratio was 4:1. Megakaryocytes were present in slightly increased numbers.

Studies were carried out to exclude a possible haemoglobinopathy. The haemoglobin electrophoretic pattern was normal, as were HbF and HbA_2 levels (1.0 and 1.9% respectively). An unstable haemoglobin was not demonstrated. The Motulsky screening tests for glucose-6-phosphate dehydrogenase deficiency done on the eighth day revealed normal activity, decolorizing within 43 min (normal 43–170 min).



Fig. 2. Scanning electron photomicrograph showing a number of pyknocytes as well as normal and mildly distorted cells. In one cell the surface membrane appears to lie in folds over the haemoglobin precipitate. × 10 000.

Pyknocytes were examined under the scanning electron microscope. Fresh blood from the patient was mixed with glutaraldehyde for fixation, the cells were packed by centrifugation, a thin smear made on a cover slip and the specimen air dried. Gold was evaporated onto the specimen in an AET/12 model vacuum coating unit to approximately 1 000 Å in depth. The cells were then examined using a Cambridge Stereoscan Mark 2A scanning electron microscope. The appearances are shown in figures 2–4.

The patient never regained consciousness and an electroencephalogram done on the fifth day showed a grossly abnormal tracing with virtual absence of any rhythmic activity. On the tenth day peritoneal dialysis was commenced but the patient died the following day. At necropsy the brain was oedematous and congested with petechial haemorrhages throughout, including the pons. The kidneys were pale and swollen and acute tubular necrosis was present. There was no obvious liver damage.

Because pyknocytosis has not previously been reported either in heat stroke or on heating erythrocytes, we attempted to induce this change *in vitro*. Blood was collected from a healthy donor into ACD (0.50 g citric acid, 1.38 g sodium citrate and 1.39 g dextrose per 100 ml). The cells were washed free of plasma and buffy layer





Fig. 3. Scanning electron photomicrograph of an erythrocyte in which the haemoglobin appears to have aggregated at one pole of the cell. It is uncertain whether the small shadows seen in the 'empty' part of the cell are membrane folds or small amounts of remnant haemoglobin. Projections of the cell membranes are clearly seen. ×40 000.

Fig. 4. Scanning electron photomicrograph of a grossly distorted cell with haemoglobin precipitated in one pole. The cell membrane appears to be discontinuous in one area. ×40 000.

by suspension in phosphate buffered saline (PBS), repeated centrifugation, removal of supernatant and resuspension in PBS. The washed cells were packed and aliquots suspended in equal volumes of PBS at the required pH. They were then incubated for 1, 4 or 6 h at temperatures of 37, 39, 41, 44 and 46 °C while shaking with glass beads. The pH of the PBS was varied between 7.0, 7.2, 7.4 and 7.6. In those instances where whole blood was used, the pH of the suspension was changed to that required by using isotonic PBS; the time of incubation and glass bead shaking was identical to that used with the washed cells. One further experiment was carried out where cells obtained from a patient with chronic renal failure (blood urea concentration 120 mg/100 ml) were washed as described before incubating them in the patient's own plasma at 37, 41 and 44 °C, at an acid pH (6.9). All these attempts at inducing pyknocytosis in vitro were unsuccessful. Normal red cells were not incubated with the patient's serum.

Discussion

Haematological parameters have been variable when measured shortly after an episode of heat stroke [2, 3, 11]. This is probably due to a variety of factors, but particularly the state of the patient's hydration and peri-

pheral circulation, and the circumstances under which heat-stroke occurred. What happens thereafter has been the subject of little systematic investigation. In an abstract published in 1955, HALDEN *et al.* [5] reported 15 patients in whom the mean haemoglobin level fell during the week following the onset of heat-stroke. There was no obvious blood loss and after carrying out red cell survival studies they concluded that anaemia was due to two factors, increased destruction of erythrocytes and failure of the bone marrow to respond adequately to the falling haemoglobin level. STEPHANINI and SPICER [13] subsequently reported one patient with haemolytic anaemia which was associated with increased fibrinolysis.

Pyknocytosis has not previously been reported in heat stroke. Pyknocytes or 'blister cells' are erythrocytes in which the haemoglobin appears to have aggregated at one pole of the cell leaving the other 'empty', an appearance which has been likened to that of a basket with a handle [8]. Pyknocytosis is rare and has only been described in association with the haemolytic phase of glucose-6 phosphate dehydrogenase (G-6-PD) deficiency [7] and sickle cell disease complicated by pulmonary infarction [8].

It is thus a phenomenon which has previously been reported only in erythrocytes which are intrinsically abnormal. In our patient haemoglobinopathy was excluded. Although the Motulsky screening test for G-6-PD deficiency was normal, this test may be unreliable during acute haemolytic episodes [12], so that G-6-PD deficiency or some other form of intrinsic abnormality may have remained undetected. That this is likely is suggested by the fact that pyknocytosis has not been produced in the many studies, including the present, in which erythrocytes have been heated *in vitro*.

The alternative possibility that pyknocytosis was induced by the combination of heat and some substance in the plasma seems less likely. The metabolic disturbances in our patient were similar to those commonly observed in heat-stroke and pyknocytosis has not previously been reported in heat stroke or induced in experimental animals [9] or human volunteers [1] by subjecting them to high temperatures. The most obvious metabolic change in our patient shortly after the onset of heat-stroke was a metabolic acidosis. However heating erythrocytes *in vitro* at varying pHs failed to reproduce the change. Repeating the experiment with uraemic serum was also unsuccessful.

The difficulty in understanding the mechanism of pyknocyte formation is compounded by uncertainty over the pathogenesis of heat injury to red

cells In this regard, postulates have varied from inactivation of key enzymes to alterations of membrane structure and function [10] It seems likely that pyknocyte formation represents a physicochemical alteration of the haemoglobin component of the erythrocyte That there may also be an effect on the cell membrane is suggested by the scanning electron micrographs which show surface membrane alterations and projections

Acknowledgements We wish to thank Mr M [illegible] of the electromicroscopy unit of the National Institute for Occupational Diseases, and Mr J [illegible] of the Diamond Research Institute for the electron microscope studies and Prof J [illegible] for his helpful advice Due acknowledgement is made to the Atomic Energy Board and the Medical Research Council for the assistance and support enabling the research concerned to be undertaken to the Director South African Institute for Medical Research for facilities granted and to the Chamber of Mines of South Africa for permission to publish this report

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Acta Haemat. 50: 257-265 (1973)

Mechanism of Action of L-Asparaginase on the Cell Cycle and Growth in Acute Lymphoblastic Leukemia[1]

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Abstract. The effect of L-asparaginase on various acute human lymphoblastic leukemia cell populations was evaluated in an investigation of its mechanism of action with respect to the blast cell cycle and the growth of the leukemic cell population. Immediately following the injection of the drug, the number of cells in S increased while those in G_2 and M decreased. The percentage in G_1 showed no appreciable change. Later a destructive effect of L-asparaginase on cells in S phase was noted, together with reduction of the size of the proliferating compartment. Reference is made to recently proposed proliferation kinetic models in acute leukemia in support of the view that the action of the drug may also be directed towards cells in transition from G_0 to G_1.

Key Words
Asparaginase
Autoradiography
Cell cycle
Cytospectrophotometry
Leukemia therapy
Lymphoblastic leukemia

L-Asparaginase is widely employed in human tumor therapy, particularly in the management of acute leukemias [8, 11, 21, 30, 31]. Its effect on the proliferative kinetics of these forms, on the other hand, has not yet been determined. It was therefore decided to examine this question with respect to certain parameters in acute lymphoblastic leukemia, since this form responds best to treatment with the enzyme. Two main questions were investigated. At what point of the mitotic cycle do the earliest kinetic changes take place? How are the gradual reduction in size of the cell population and the loss of its proliferative activity brought about?

Materials and Methods

Five cases of acute lymphoblastic leukemia were studied. Four had received no previous treatment, one patient was in relapse 15 days after suspension of associated

[1] Supported by CNR, Roma, [illegible]

methotrexate vincristine and prednisone management. Peripheral white cell values ranged from 40 000 to 750 000/mm³ (lymphoblasts 84–91%). Secondary anemia, adenopathies, and/or more or less pronounced liver and spleen enlargement were constant. Hemorrhagic diathesis and oropharyngeal ulcers and necroses were occasional findings. Each patient received a single dose of 100–200 U/kg asparaginase (Crasnitin, Bayer) per day. The administration pattern is shown in figure 1. Marrow samples were taken by means of a normal technique and passed through thin needles; the disaggregated cells were counted. The following marrow and/or peripheral blood parameters were determined prior to and at various times after the commencement of the treatment: cell counts per mm³, percentage lymphoblasts, mitotic index (on at least 3 000 cells), mean lymphoblast diameter. On each occasion an aliquot was diluted with Hank's solution (2:1 or 1:1 according to cellularity) and incubated with 1–2.5 µCi/ml ³H thymidine (specific activity 10 Ci/mM) for 30 min at 37 °C. After fixing in Carnoy's fluid the smears were covered with Ilford K_2 emulsion and exposed for 10–15 days at 4 °C. Development and fixing of the slides was followed by staining with Giemsa buffered to pH 7.4.

Labelling index (LI) and mean grain count (m.g.c.) values were determined on at least 3 000 cells and 100–200 cells, respectively.

A microspectrophotometric method was used to evaluate cell DNA contents in cases 1, 3, 4 and 5, using photographic maps of Giemsa stained slides. Each cell photographed was assigned a number and its diameter was determined. After destaining, the smears were subjected to Feulgen staining. DNA content values were then obtained for each numbered cell using a Barr and Stroud GN2 microdensitometer. The monochromatic light wavelength employed was 5 400 Å, and the values of absorbance were expressed in arbitrary units.

The slides were then autoradiographed as described and cells with a thymidine uptake were located on the maps. At least 400 cells were subjected to combined spectrophotometric and autoradiographic study on each occasion. Lastly, the method of GAVOSTO *et al.* [17] was used to determine the growth fraction (GF) from the relationship between LI and cell diameter. With this method, the size of the proliferating compartment is determined by simultaneous evaluation of the percentage of blast cells of different diameters and their respective labelling indices.

Results

Clinical and hematological response to treatment. A positive response was noted in all 5 cases. This included an improvement in the clinical picture, with reductions in spleen and lymph node size and the disappearance of hemorrhages and fever. Changes in the blood picture were the first signs of the effectiveness of the treatment and included rapid falls in both peripheral and marrow lymphoblasts (fig. 1).

In cases 4 and 5, where frequent peripheral blood samples were obtained, blasts decreased after 6 and 7 h, respectively. As can be seen in figure 3, peripheral LIs were higher than those in bone marrow. This had

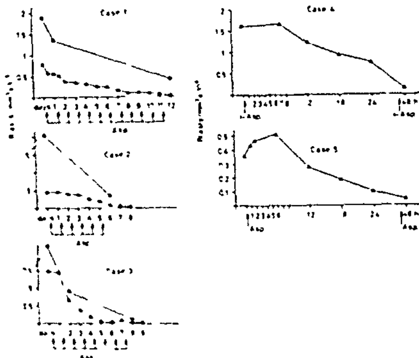


Fig. 1. Number of bone marrow and peripheral blasts before and during therapy with L-asparaginase. ● = Bone marrow blasts, ▲ = peripheral blasts.

been occasionally noted in infantile ALL [18, 19], as well as in many cases of disseminated lymphosarcomatosis, where LI, MI, GF, and large blast percents were higher in peripheral blood. This was therefore not considered as an end compartment, as usual, but as one formed by an actively proliferating lymphoblastic population. Cases 4 and 5 presented all these features, and the kinetic parameters for peripheral blasts were followed.

Variations in LI, GF and m.g.t. Marked falls in both peripheral and marrow LI values were noted 24 h after injection (Fig. 2 and 3). Cases 4 and 5 presented high peripheral LIs and frequent determinations showed an increase in the percentage of labelled cells soon after asparaginase, this was slight in case 4, though well marked and progressive to up to 3 times the initial value in case 5.

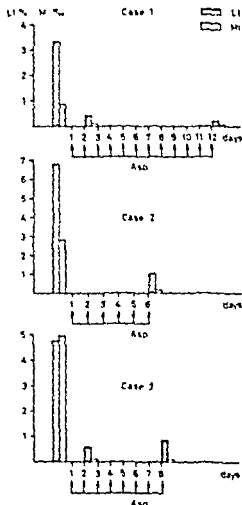


Fig 2 Bone marrow labelling index (LI) and mitotic index (MI) before and during therapy with L asparaginase in cases 1–3

MI values fell to almost zero within a few hours When LI values increased, this decrease was noted very quickly M g c values were obtained in cases 4 and 5 Gradual reduction took place

Changes in lymphoblast diameter showed that decreased LI values were accompanied by a decrease in the number of larger cells, resulting in a population composed of small cells with a relatively restricted spread of diameter values GF values decreased progressively, though an initial increase in LI was accompanied by higher GF values (table I)

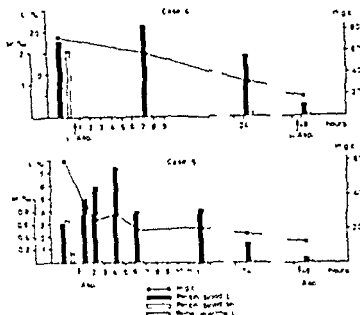


Fig. 3. Peripheral blood labelling index (LI), mitotic index (MI) and m.g.c., before therapy bone marrow labelling index in cases 4 and 5

Lymphoblast distribution in different phases of the cell cycle. Sequential determination of DNA spectrophotometric values and ^{3}H-thymidine uptake was used to evaluate the distribution of lymphoblasts in different stages of the cell cycle prior to and during treatment. Figure 4 shows the DNA content of labelled and unlabelled blasts of case 4, while table I illustrates the cell distribution patterns in all cases. Before administration of the drug cells with diploid DNA were unlabelled and thus represent the population fraction in G_1 or G_0 + Z. Apart from a small percentage in G_2 (2–10%) with a DNA content of 4C), all cells with greater than 2C DNA were labelled.

Cells with more than 2C DNA content decreased in number after the commencement of therapy in cases 1 and 3. In cases 4 and 5, on the other hand, early increases in the number of such cells paralleled the enhanced LI values, coupled with percent reductions in cells in G_2 and M. These findings are seen as an expression of the accumulation of cells in S, and a

Table I Distribution of blasts in the phases of cell cycle and growth fraction (GF) before and during therapy (values in ‰)

Experimental time	Actually proliferating blasts					Non proliferating blasts $(G_0+Z)=(1\text{-}GF)$	GF[1]
	G_1	S	G_2	M	U		
Case 1							
Before therapy	333	35	11	1	0	620	0.38
48 h after L-asp	93	2	5	0	20	580	0.12
Case 3							
Before therapy	313	47	10	5	0	625	0.37
48 h after L-asp	134	5	4	0	1	856	0.14
8 days after L-asp	3	10	5	0	2	980	0.02
Case 4							
Before therapy	429	184	23	2	2	360	0.64
7 h after L-asp	463	220	6.5	0.5	0	310	0.69
24 h after L-asp	474	155	9	0	2	360	0.64
48 h after L-asp	235	16	10	0	9	730	0.27
Case 5							
Before therapy	348	25	6	1	0	620	0.38
4 h after L-asp	349	73	7	0	1	570	0.43
24 h after L-asp	180	17	10	0	23	770	0.23

Abbreviations: G_1 = unlabelled cells with 2C DNA content, S = ^3H thymidine labelled cells with greather than 2C DNA content. G_2 = unlabelled cells with 4C DNA. M = mitotic index. U = unlabelled cells with DNA content between 2C and 4C.

[1] GF is expressed as a fraction of 1

slowing down of the passage of cells through this phase. After the 6th and 24th h, in fact, the number of cells in S (>2C DNA) fell to about 1%.

Unlabelled cells with DNA contents of over 2C and less than 4C (U cells) appeared at the 48th h in cases 1 and 4, and at the 24th h in case 5. These cells may be viewed as blocked in S phase.

Discussion

In acute lymphoblastic leukemia treated with L-asparaginase, the drug brings about chanees in certain kinetic parameters and a redistribution of blasts throughout the different staees of the cycle at a very early stage, before any reduction in cell numbers takes place.

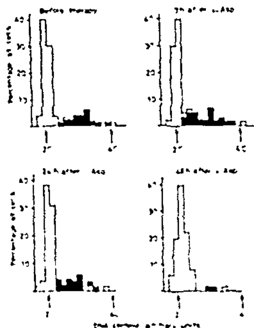


Fig. 4. DNA content of labelled and unlabelled blasts in case 4 before and during therapy

A gradual increase in ³H thymidine LI values was noted within the first 4–7 h in 2 cases. The mitotic index showed a marked downward course. Cells with more than 2C DNA displayed a percent increase parallel with that in the LI, accompanied by a fall in the number of unlabelled 4C cells. GI values were virtually unaffected. These data were used to calculate the percent distribution of the proliferating population over the different phases of the cell cycle. Immediately following the injection, the number of cells in S increased, while those in G_2 and M decreased. The percentage in G_1 showed no appreciable change.

L-Asparaginase seems to be responsible for a very prompt slowing down of the passage of cells through the S phase, coupled with their accumulation and blocked exit from this phase. The fact that the passage from G_1 to S is not blocked at this stage can be deduced from the increase in LI values. The slowing down of the passage through S is also attested by the gradual reduction in m.i., in step with the increase in LI. This pro-

gressive fall-off in the uptake of ^3H-thymidine and its incorporation in DNA points to a decrease in the synthesis of DNA itself. A depression of DNA synthesis rate was already observed in 6C3HED lymphoma cultures added with L-asparaginase [6, 12].

An alternative explanation for the increase in LI values would be that L-asparaginase is directly responsible for damage to the already synthesized DNA chain, so that repair processes are at work at an early stage, before cell death occurs [13]. Yet this view is in conflict with the cytospectrophotometric demonstration of increased mean DNA contents running parallel with increase in LI values, and the uniform distribution of labelled cells between 2C and 4C DNA. By the same token, there is no sign of a DNA repair peak in the grain count curves.

The 24th and 48th h specimens presented evidence of serious damage to the proliferative capacity of the blast cells. The labelling data made it clear that there had been a marked fall in the percentage of cells capable of DNA synthesis. A 7- to 50 fold drop in LI values was coupled with a fall in the number of cells with greater than 2C DNA. GT values were much reduced and the MI was 0 in all cases.

In case 5, these changes were already apparent at the 6th h and thereafter became increasingly evident. When the first signs of clinical remission appeared at these later experimental times, there was a marked reduction in the leukemic cell population in all cases. Both marrow and peripheral cellularity were diminished and there was a fall in blast percents.

The residual population was homogeneous in a number of respects: diameters were small and lay within a narrow range, DNA values were 2C in nearly all cells. This means that nearly all the remaining cells probably are in a resting stage.

The effect of a single high dose of L-asparaginase (1,000–2,000 U/kg) has been studied by LAMPKIN *et al.* [24] and SAUNDERS [28] in 1 and 5 cases, respectively. LAMPKIN noted a slight increase in LI after 1 h and a marked fall after 24 h, whereas MI values fell from the 1st h onwards. SAUNDERS observed a constant fall in LI values from the 6th h on, while MI values fell both later and more slowly. Attention must be drawn to the fact that SAUNDERS did not take the immediate post-injection picture into account. A further explanation for the discrepancy between his results and our cases 4 and 5 may be seen in his employment of a 20 fold higher dose of L-asparaginase in a single injection. We observed a cytolytic effect at a later period and it is at this point that the discrepancy between the two sets of data disappears. It may be suggested that the lower asparaginase doses, extensively employed for treatment, permit the detection of

continuously pass from G_0 to feed the proliferating compartment are activated

Asparaginase-induced inhibition of the passage from G_0 to cycle has been demonstrated in two experimental models (blastogenesis of human lymphocytes [1, 26, 29], liver regeneration in the partially hepatectomised rat [2–4]) Our data suggest that asparaginase most probably act also at this level in lymphoblastic leukemia Several days after the commencement of treatment morphologically and cytochemically homogeneous lymphoblasts were still present in considerable number It can hardly be suggested that these were only cells in Z phase, since earlier works has shown that survival times in this compartment are of the order of no more than a few days [15, 22] One can therefore suppose that these, too were blasts in G_0 phase whose reentry into the proliferating compartment was being markedly inhibited as was clear from the extremely low LI and GF values

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Acta Haemat. 50: 269–274 (1973)

The Effect of L-Asparaginase on DNA and RNA Synthesis by Lymphoblasts of Acute Lymphocytic Leukemia[1]

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Abstract. Bone marrow cells of 8 patients with acute lymphocytic leukemia were studied by cytochemical and autoradiographic methods for the synthesis of nucleic acids in the presence or absence of L-asparaginase. The cultures included thymidine for DNA or uridine for RNA metabolism. In the absence of the enzyme the leukemic lymphoblasts reacted more intensely for DNA and RNA synthesis. The addition of the enzyme revealed depressed incorporation of the labeled precursors by the primitive cells.

Key Words
Asparaginase
Bone marrow culture
Cytochemistry
Autoradiography
DNA and RNA synthesis
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L-Asparaginase is a known inhibitor of the blastic transformation of lymphocytes *in vivo* as well as *in vitro* [2, 9]. It appears to exert its effects by depriving susceptible cells of exogenous L-asparagine which results in the inhibition of protein synthesis [1, 3, 4]. Associated with the inhibition of protein synthesis is the repression of nucleic acid metabolism as shown by experiments with some animal tumors [6].

In the present investigation, the effect of L-asparaginase on nucleic acid synthesis was studied cytochemically [5, 8] to characterize the intensity of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) reactivity. Moreover, to evaluate the inhibitory activity of L-asparaginase in DNA and RNA metabolism, the percentage of large blast cells incorporating thymidine and uridine in the presence or absence of the enzyme was determined.

[1] Supported in part by Institutional Research Grant No. [illegible] to the University of Michigan from the American Cancer Society.

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Materials and Methods

Bone marrow cultures of 8 patients with acute lymphocytic leukemia (ALL) were prepared and included Spinner modified Eagle's minimum essential medium supplemented with L-glutamine, fetal calf serum, phytohemagglutinin, and L-asparaginase. Cultures without the enzyme were also processed. The cultures were incubated with the addition of thymidine or uridine. The epon embedded specimens were dipped in Kodak NTB₂ and developed with Kodak D19ₐ developer. The slides were stained with Giemsa stain and evaluated by counting 300 large lymphoblasts from each specimen for heavily labeled, lightly labeled, and nonreactive cells.

Results

The primitive cells of ALL stained by the Feulgen reaction for DNA tended to stain more intensely with many cells possessing discernable, larger aggregates of chromosomal bodies than similarly stained cells incubated in cultures containing L-asparaginase (fig. 1a, b). Likewise, the primitive cells of ALL following the methyl-green-pyronin method for RNA appeared to be more reactive than leukemic lymphoblasts cultured in the presence of L-asparaginase prior to staining (fig. 1c, d).

Thymidine labeling for DNA synthesis showed many of the leukemic lymphoblasts to be heavily labeled with fewer nonreactive cells; similar specimens treated with L-asparaginase and thymidine demonstrated more nonreactive lymphoblasts and fewer lightly and intensely labeling cells (table I, fig. 2a, b). RNA metabolism using uridine in the presence of L-asparaginase revealed depressed incorporation of the label into the leukemic lymphoblasts with more cells appearing nonreactive and fewer cells showing intense reactivity. In comparison, without the enzyme, cellular incorporation of uridine exhibited many heavily and lightly labeling cells with fewer nonreactive lymphoblasts (table I, fig. 2c, d). With both thymidine and uridine, background labeling was light.

Discussion

L-Asparaginase is used as a chemotherapeutic agent in acute leukemias and malignant animal and human tumors because of its antitumor activity. The cells in various tumors are found to be asparaginase-sensitive or asparaginase-resistant. Asparaginase-resistant cells have high levels of the enzyme, asparagine synthetase, and have the ability of making asparagine



Fig. 1. [illegible] cells of acute lymphoblastic leukemia. Feulgen reaction: *a* Intense nuclear activity with visible aggregates of chromosomal bodies in a lymphoblast from cultures without L-asparaginase. *b* A few visible chromatin granules in a lymphoblast cultured with L-asparaginase. Methyl green-pyronin staining: *c* Intense cytoplasmic reaction in lymphoblasts from cultures without L-asparaginase. *d* Light cytoplasmic reaction in lymphoblasts incubated with L-asparaginase. × 1,500

Table I. The incorporation of thymidine and uridine by lymphoblasts of acute lymphocytic leukemia cultured in the absence or presence of L-asparaginase and expressed as the average percentage of 500 cells

	Without L-asparaginase			With L-asparaginase		
	Heavily labeled	Lightly labeled	Non-reactive	Heavily labeled	Lightly labeled	Non-reactive
Thymidine	41.9	39.0	19.1	2.2	23.9	[illegible]
Uridine	51.2	[illegible]	9.1	2.8	[illegible]	[illegible]

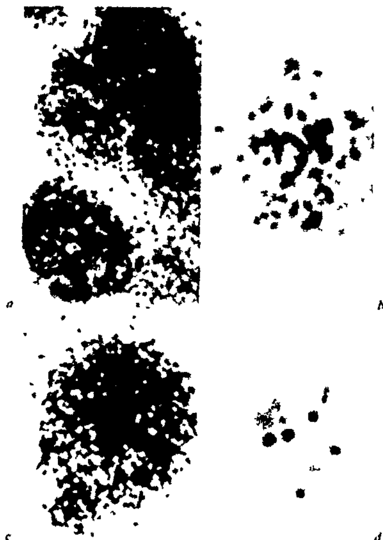


Fig. 2. Primitive cells of acute lymphocytic leukemia. Thymidine labeling: *a* Heavier labeling in lymphoblasts from cultures without L-asparaginase. *b* Lighter labeling in a lymphoblast cultured with L-asparaginase. Uridine labeling: *c* Heavier labeling in a lymphoblast incubated without L-asparaginase. *d* Lighter labeling in a lymphoblast cultured with L-asparaginase. × 1,500.

in sufficient quantities for their own metabolic requirements [11]. The sensitive cells are deficient in asparagine synthetase [7, 11, 13] and, when deprived of asparagine by L-asparaginase, show a depression of protein metabolism since they cannot synthesize adequate amounts of the amino acid. For growth, the sensitive cells must depend on the asparagine present

in the blood which in normal human plasma is reported to be 0.5–0.7 mg/100 ml [9]. Likewise, in order to grow in culture sensitive cells require the addition of L-asparagine to the culture media.

A recent investigation indicates that Jensen sarcoma cells in the presence of L-asparaginase are repressed in their synthesis of DNA, probably resulting from the depression of protein synthesis which is considered essential for the initiation of DNA metabolism [10]. Once initiated, protein synthesis may or may not be required to maintain DNA synthesis [12]. Mouse lymphomas depleted of asparagine by L-asparaginase demonstrate the inhibition of all types of RNA. The depletion of the amino acid may cause the selective inhibition of the synthesis of ribosomal RNA followed by the depression of messenger RNA and finally transfer RNA metabolism [6].

In the present study the repression of DNA and RNA synthesis in the primitive cells of ALL, evaluated cytochemically and by the incorporation of labeled precursors in the presence of L-asparaginase, tends to agree with the results of these investigations [6, 10, 12]. In cultures with L-asparaginase, the disappearance of heavily labeled cells points to the decreased rate of DNA and RNA synthesis, suggesting that the effect of L-asparaginase on DNA and RNA synthesis is secondary to the repression of protein synthesis.

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Acta Haemat. 50: 275–283 (1973)

Lymphocyte Surface Markers in Lymphoproliferative Disorders

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Abstract. Peripheral blood lymphocytes of [illegible] normal controls and 32 patients with lymphoproliferative disorders were examined for the presence of surface markers. T cell frequency was decreased in chronic lymphocytic leukemia (CLL), Hodgkin's disease and myeloma, and was normal or decreased in acute lymphocytic leukemia (ALL). Lymphocytes with surface membrane receptors specific for all Ig were markedly increased in lymphosarcoma and cryomacroglobulinemia, extremely decreased in ALL, normal in Hodgkin's disease; only lymphocytes specific for IgM were increased in CLL. Receptors for C3 were diminished or absent in CLL and ALL. In the remission phase of ALL, EAC and E rosettes returned to normal and sIg were almost normal. The approach presented in this article suggests a classification of these diseases in relation to the T and B cell frequency which may be useful for diagnosis and therapeutic implications.

Key Words
EAC rosettes
E rosettes
Immunofluorescence
Immunoglobulins
Lymphoproliferative diseases
Surface markers

Lymphocytes are divided into two major groups according to their origin and function. T lymphocytes derived from the thymus [3, 16] are concerned with cellular immunity [15], and B lymphocytes derived from an unknown site homologous to the bursa of Fabricius in birds are concerned with humoral antibody production [2, 31]. These cells can further be differentiated according to specific surface markers which permit them to be related to cellular or humoral functions [9, 10, 18].

Surface markers are used to measure the frequency of T and B cells and have also been used to describe different stages of lymphoid cell differentiation [25, 27, 28]. B cells can be identified by the presence of membrane surface immunoglobulins (sIg) [11, 21, 30, 32], which bind specific heavy

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Abstract. Peripheral blood lymphocytes of 18 normal controls and 32 patients with lymphoproliferative disorders were examined for the presence of surface markers. T cell frequency was decreased in chronic lymphocytic leukemia (CLL), Hodgkin's disease and myeloma, and was normal or decreased in acute lymphoblastic leukemia (ALL). Lymphocytes with surface membrane receptors specific for all Ig were markedly increased in lymphosarcoma and macroglobulinemia, extremely decreased in ALL, normal in Hodgkin's disease; only lymphocytes specific for IgM were increased in CLL. Receptors for C3 were diminished or absent in CLL and ALL. In the remission phase of ALL, EAC and E rosettes returned to normal and sIg were almost normal. The approach presented in this article suggests a classification of these diseases in relation to the T and B cell frequencies which may be useful for diagnostic and therapeutic implications.

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Surface markers are used to measure the frequency of T and B cells and have also been used to describe different stages of lymphoid cell differentiation [25, 27, 2[illegible]]. B cells can be identified by the presence of membrane surface immunoglobulins (sIg) [11, 21, 30, 32], which bind specific heavy

chain antiserum, their characteristic receptor for C3 complement component [5, 8] and their ability to bind certain antigen-antibody complexes [4]. T cell frequency can be measured by their spontaneous formation of rosettes with sheep red blood cells [6, 13, 19] and more recently by their ability to react with specific human anti-T serum [2].

The present study is concerned with determining the frequency of T and B cells in lymphoproliferative diseases through the use of surface markers specific for B cells – sIg and receptors for C3-activated complement component (EAC test) – and T cells, the spontaneous formation of rosettes with sheep red blood cells (E test).

Materials and Methods

Patients. Normal controls were laboratory personnel, medical students, and their respective relatives. All controls were examined according to accepted clinical and laboratory criteria including estimation of the immunoglobulin serum levels, and were found to be normal in every respect.

32 patients with lymphoproliferative disorders consisted of 3 treated and 7 untreated cases of chronic lymphocytic leukemia (CLL), 1 case of primitive cryoglobulinemia, 1 case of lymphosarcoma, 6 cases with Hodgkin's disease, 5 cases with multiple myeloma, 20 cases with benign monoclonal hypergammaglobulinemia and 7 cases with acute lymphocytic leukemia (ALL). Three of the patients with ALL were examined in the acute phase and had not received previous treatment, while the other 4 had been treated and were asymptomatic in remission. The diagnosis of the lymphoproliferative disorders was based on accepted clinical and laboratory criteria, including bone marrow and lymph node biopsies in all cases, and isolation and typing of the monoclonal components in the serum and urine in the cases of myeloma.

Lymphocyte preparations and immunofluorescent staining. Lymphocytes were isolated on a Ficoll isopaque gradient [28], washed 3 times in gelatin veronal buffer (GVB), and stained by membrane immunofluorescence [14] by incubation with fluorescein-conjugated antiserums specific for the different heavy chain immunoglobulin classes. An ultraviolet microscope with vertical illumination was used to identify cells positive for membrane immunofluorescence [23].

Test procedure for E rosettes. The test for E rosettes was performed by incubating lymphocytes with sheep red blood cells (SRBC) for 15 min at 37°C, then overnight at 4°C [13]. 200 lymphocytes were counted, those binding more than 3 SRBC were considered positive.

Test procedure for EAC rosettes. The test for EAC rosettes was performed according to JONDAHL *et al.* [13]. 0.25 ml of SRBC previously treated with rabbit IgM anti SRBC and human complement were mixed with an equal volume of lymphocyte suspension in GVB and EDTA at 37°C for 15 min [23]. 200 lymphocytes were counted, those binding more than 3 SRBC were considered positive.



Fig. 1. SIg detected by immunofluorescence showing a characteristic uneven or granular fluorescence on the greater part of the cell surface.



Fig. 2. Formation of rosettes with lymphocytes binding more than 3 SRBC.

Results

The positive cells observed by immunofluorescence showed a characteristic uneven or granular fluorescence on the greater part of the cell surface (fig. 1); negative cells showed no detectable fluorescence. Concerning the lymphocytes in the normal controls, [illegible]2% reacted with the fluorescein conjugated antiserum specific IgG, 4.3% with anti-IgA, 8.9% with anti-IgM, 0.9% with anti-IgD and 0.7% with anti-IgE (table I). Cells specific for the formation of rosettes were considered positive with more than 3 SRBC on their membrane (fig. 2). The percentage of normal lymphocytes forming EAC rosettes was 21.4 whereas 52.4% formed E rosettes (table I).

Table I Surface markers on normal peripheral blood lymphocytes

Subjects	Surface immunoglobulins[1]					EAC[2] rosettes	E[3] rosettes
	G	A	M	D	E		
V.A	7	3	8	0	0	24	46
S C	6	2	7	0	0	34	69
M.A	12	4	16	1	4	33	50
D A R	10	6	6	0	0	30	50
D A C	8	6	6	0	0	18	54
L.E	4	6	10	2	1	30	47
F A	8	8	12	1	2	–	–
L.E	2	4	8	1	0 5	21	61
M O	12	8	10	3	0	22	40
D.A	8	2	4	5	2	22	43
S A	3	4	8	1	0	–	50
C M V	6	2	8	0	–	21	57
V M	5	4	5	0 5	0 5	25	50
L.G	12	4	12	0	0	13	57
S E	7	1	10	0	0	23	50
G S	12	4	12	0 5	0 5	23	53
P F F	4	8	10	0	0	26	43
L.V	3	1	9	1	1	23	52
Mean	7 2	4 3	8 9	0 9	0 7	23 4	52.4
Range	2–12	1–8	4–16	0–5	0–4	13–34	40–69
SD	3 27	2.29	1 17	1 00	1 00	5 74	7 28

[1] Values are expressed in percentage of the cells positive for membrane immunoglobulins

[2] EAC = lymphocytes which form rosettes with sheep red blood cells coated with anti-sheep rabbit erythrocyte of class IgM plus human complement

[3] E = lymphocytes which spontaneously form rosettes with SRBC

The results pertaining to patients with lymphoproliferative disorders are reported in table II In untreated CLL, sIg were normal for IgG and IgA and significantly increased for IgM and IgD Only in 2 cases was there evidence of IgE The formation of EAC and E rosettes was significantly decreased In treated CLL, lymphocyte sIg were normal or decreased except for IgM which was increased, though not to the extent observed in the untreated cases EAC and E rosettes were significantly decreased In the cases of CLL complicated with agammaglobulinemia, the percentage of lymphocytes positive for sIg was decreased except in 1 case which had an increase in IgM and normal IgG EAC rosettes were low and E rosettes normal In

Table II Surface markers on lymphocytes of patients with lymphoproliferative disorders

Case No	Diagnosis		Lymphocytes with surface Ig % G	A	M	D	I	EAC ros %	E ros %
1	CLL u		6	0	48	3	0	0	13
2	CLL u		8	6	58	3	0	1	11
3	CLL u		4	3	78	11	4	11	18
4	CLL u		8	4	52	6	2	6	11
5	CLL u		10	6	65	4	1	14	2
6	CLL tr		2	6	12	0	0	5	24
7	CLL tr		6	2	34	9	0	6	42
8	CLL t		2	8	41	0	0	9	39
9	CLL w		6	3	26	0	0	12	56
10	CLL w		2	0	0	0	0	1	49
11	CMG		12	20	66	4	0	20	55
12	L		[illegible]	16	14	2	4	–	44
13	Hodgk	[illegible]	[illegible]	6	2	–	–	27	46
14	Hodgk	[illegible]		2	10	–	–	30	34
15	Hodgk	[illegible]		0	0	0	0	2	34
16	Hodgk	[illegible]		2	6	0	0	19	39
17	Hodgk	[illegible]		2	4	2	0	16	32
18	Hodgk	[illegible]		2	10	–	–	30	34
19	M IgG			2	8	0	0	54	51
20	M IgG			4	2	0	0	45	27
21	M IgG			9	6	2	0	25	29
22	M IgA			0	0	0	0	29	32
23	M IgA			2	6	0	0	–	–
24	BMH	[illegible]		2	6	0	0	39	73
25	BMHL	[illegible]		1	4	0	0	8	45
26	ALL a	[illegible]		1	3	–	–	12	31
27	ALL a	[illegible]		[illegible]	0	0	0	2	28
28	ALL a	[illegible]	[illegible]		3	0	0	8	92
29	ALL a	[illegible]	1		4	–	–	3	27
30	ALL re	[illegible]	1		4	–	–	27	64
31	ALL rem	[illegible]	[illegible]		4		–	31	45
32	ALL rem	[illegible]	[illegible]		[illegible]		–	28	61
33	ALL rem	[illegible]	1		4		–	36	45

[illegible]

the case with CMG, all sIg were increased IgG slightly, and IgA, IgM and IgD markedly The EAC and E tests were normal In the case of lymphosarcoma all sIg were significantly elevated The EAC test was not performed and the E test was normal In Hodgkin's disease the sIg were normal except in 1 case which was reduced EAC rosettes were normal except in case 15, and all had reduced E rosettes

In multiple myeloma, sIg were normal or decreased, only case 22 showed complete absence of IgA and IgM EAC tests were normal or increased and E tests were decreased In benign monoclonal hypergammaglobulinemia, sIg for IgG and IgM were normal and IgA was decreased EAC tests were normal in one and markedly reduced in the other, E tests were normal

In untreated ALL, all sIg were extremely low In case 28 IgG and IgA were absent and in case 27 all sIg were absent The formation of EAC rosettes was very low and E rosettes were normal or decreased In the patients in remission sIg were similarly reduced, however, not to the extent found in the untreated cases EAC and E tests were normal in 4 of the 5 cases Peripheral blood smears of the patients in remission did not show any lymphoblasts or atypical cells

Discussion

The analysis of sIg EAC and E rosettes on peripheral blood lymphocytes of patients with lymphoproliferative disorders has provided us with information concerning the frequency of T and B cells In comparing our results with those of others [1, 26] we found that sIg levels for IgM in untreated CLL are markedly increased whereas in the treated cases and in the patients with associated agammaglobulinemia IgM levels were only moderately increased however, in comparison to the untreated, they are significantly reduced The difference observed by others in similar patients is probably correlated with the number of peripheral blood lymphocytes or the stage of the disease or treatment [1, 20 24 26] An established explanation as to the origin of the type B lymphocytes with sIg specific for IgM has not been provided In cases 2 5 of untreated CLL, we measured EAC rosettes using mouse complement the respective percentages were 28 16 5 and 20 There was a significant increase in the number of lymphocytes forming EAC rosettes in only one case This is not in agreement with Pincus *et al* [22] who found high levels of EAC rosette formation with mouse complement in CLL This discrepancy can be due to different technique measurement of patients

in different stages of the disease or the different modulation patterns in the membrane of pathologic lymphocytes which produce different surface receptors for human and mouse complement. The low level of E rosettes is in agreement with the reduced cellular immunity in both *in vivo* and *in vitro* studies performed by others [7, 17].

In cryoglobulinemia and lymphosarcoma, there was an increase in lymphocytes specific for IgG, IgA and IgM. Present experience leaves us unable to come to a valid explanation as to their origin; however, we can conclude that there is a possible defect in maturation or an imbalance in Ig synthesis and secretion (synthesis being greater than secretion).

In Hodgkin's disease, B cell frequency is normal whereas T cell frequency is decreased. This is in agreement with the defect in T cell function observed by others with *in vitro* PHA response [12]. It is interesting to note that in myeloma there is a partial defect in B lymphocytes related to sIg which is variable and not specific for the particular type of myeloma. These results agree with those of Cooper [personal commun.] in 18 cases of myeloma. On the contrary, the lymphocytes forming EAC rosettes are increased which confirms the independence of two populations of lymphocytes or the possibility of the same cell having an increase in one or both receptors.

It is interesting to note the different patterns of lymphocytes with membrane receptors in the treated and untreated cases of chronic and acute leukemia. Lymphocytes in untreated cases of ALL lack surface Ig, have decreased EAC rosette and decreased or normal E rosette formation. This indicates that in some cases of ALL, T cells are the dominant population while in others the lymphocytes belong neither to T nor B cell populations. In remission there is a low level of sIg and normal formation of EAC and E rosettes. This reversion toward normalcy indicates that it is possible to follow the course, measure the degree of severity and foresee the prognosis in these patients, and possibly in other lymphoproliferative disorders, by simply measuring the frequency of lymphocytes through the use of surface markers.

Lymphoproliferative diseases have individual characteristic patterns of lymphocytes with surface markers, and with a working knowledge of these patterns one would be able to confirm the clinical diagnosis and evaluate the severity of lymphoproliferative diseases. Further study at different stages of these diseases is at present desirable. With the identification of other markers, we foresee the possibility of using the identification of surface markers not only as a routine examination for the early detection of cancer, but also for the evaluation of the stage and the progress of the disease.

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Acknowledgements This work was supported by a grant from the Consiglio Nazionale dell Ricerche in Rome, Italy We wish to thank Dr H WIGZELL for his instruction in the techniques used and for preliminary communications, and Profs PAPA and FONTANA for referring the patients with lymphocytic leukemia

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Acknowledgements. This work was supported by a grant from the Consiglio Nazionale dell Ricerche in Rome, Italy. We wish to thank Dr. H. WIGZELL for his instruction in the techniques used and for preliminary communications, and Profs. PAPA and FONTANA for referring the patients with lymphocytic leukemia.

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Table I. Test subjects with different diseases

Patient No.	Age, years	Sex	Diagnosis
10	22	w	epilepsia
11	64	w	epilepsia
12	50	w	epilepsia
13	30	w	hyperthyreosis gravis
14	24	w	hepatitis infectiosa
15	63	m	uraemia gravis, degen. polycystica ren.
16	58	m	cirrhosis hepatis
17	22	w	lymphogranulomatosis maligna
18	72	w	myeloma multiplex
19	49	w	morbus collagenosis
20	17	m	ex eczema [illegible] acne vulgaris

The table excludes No. 1–9, the young and healthy control subjects. Numbering of subjects 10–20 is the same as in table III.

therefore, not suited for the study of extensive materials or for mass working.

After some initial technical difficulties we have been able to start collecting control material and, along with it, type cases from every pathological sector. Prior to the present paper, we have been able to show preliminarily that the protein binding of FAA in the course of normal pregnancy undergoes changes in that FAA binding to proteins, and primarily to transferrin, increases. At the same time the binding to α_2-macroglobulin decreased. Very soon after delivery the FAA was found to have disappeared from the transferrin zone [5].

The administration of diphenylhydantoin (DPH) is well known to be connected with a fall in the FAA content of the serum. It has been assumed that the medication caused the FAA deficiency. To date it has not been possible to explain this phenomenon, and it has been attributed to unknown factors, often to a disturbed absorption of FAA from the gastrointestinal tract [2]. In the following, we will describe the effect of DPH on the binding of FAA to serum proteins. At the same time, findings concerned with some [illegible] diseases will be presented, showing that many factors affect the FAA metabolism. How specific to each factor these changes are can probably be ascertained by later extensive [illegible]ies.

Table II. Protein and FAA distributions of the healthy control subjects in chromatography

Protein zones	Mean	SD	Range
Whole serum			
Proteins, mg/ml	78.3	6.7	70.8–88.4
FAA, ng/ml	4.1	1.3	3.0–5.8
FAA/protein, ng/g	20.6	5.2	11.6–27.2
Protein-bound FAA, %	41.0	19.5	22.0–82.0
Zone I			
Proteins, %	13.0	2.8	10.0–17.0
FAA, %	3.0	2.6	0.0–8.0
FAA/protein, pg/mg	4.0	4.3	0.0–12.0
Zone II			
Proteins, %	7.0	1.4	5.0–9.0
FAA, %	21.0	8.4	10.0–39.0
FAA/protein, pg/mg	65.0	49.0	40.0–185.0
Zone III			
Proteins, %	2.0	1.3	1.0–4.0
FAA, %	12.0	3.0	8.0–17.0
FAA/protein, pg/mg	129.0	76.0	63.0–261.0
Zone IV			
Proteins, %	7.0	2.2	5.0–11.0
FAA, %	41.0	16.6	29.0–63.0
FAA/protein, pg/mg	122.0	35.8	72.0–167.0
Zone V			
Proteins, %	70.0	3.3	64.0–76.0
FAA, %	22.0	11.4	7.0–41.0
FAA/protein, pg/mg	6.0	3.3	3.0–13.0

The ordinal numbers I–V of the protein zones are the same as in figures 1–4.

Material and Methods

The control series consisted of 9 healthy students and sportsmen, 7 men and 2 women, aged 15–26 (mean 23) years. The total results are shown in table II and the type chromatograms in figures 1 and 2.

The series of the patients studied consisted of 11 test subjects, with characteristics shown in table I. All patients were examined at the University Clinics by the relevant specialists. All the facilities afforded by clinical chemistry and a roentgen laboratory, and by serologic and cytologic methods were used in making the diagnosis. An effort was made to choose the present test subjects from among the

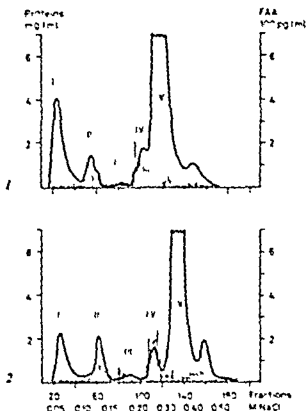


Fig 1 Typical picture of the chromatography of the serum of a healthy woman Vertical lines indicate FAA Protein zones are numbered from I to V

Fig 2 Typical picture of the chromatography of a healthy man Symbols are the same as for figure 1

socalled type cases whose clinical diagnosis was clear cut and certain At the time the specimen was taken for chromatography the patient had not yet received any treatment and was in his natural pathological state

Specimens of venous blood were taken for DEAE Sephadex chromatography in the morning after an overnight fast Blood samples for DPH determination were simultaneously taken from patients No 10 12 (table I) The DPH contents were No 10 0.7 mg 100 ml No 11 1.6 mg 100 ml and No 12, 2.6 mg 100 ml All 3 patients had taken 300 mg DPH daily for more than a year

The chromatography and associated laboratory procedures have been described in detail previously [5] The FAA of the serum and the chromatography fractions was determined by the L cases method [4]

The results of chromatography for the normal series are presented in table II and for the test series in table III The type illustrations of the chromatograms of normal subjects are shown in figures 1 (woman) and 2 (man) those of the test patients in figures 3 (DPH users) and 4 (osteomyelitis)

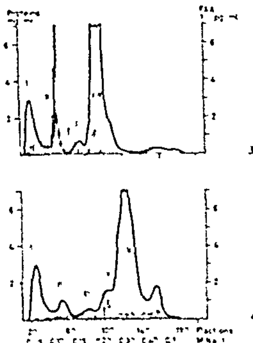


Fig. 3. Chromatography of the serum of a DPH user. Symbols as in figure 1. The subject (No. 11 of table I) had 300 mg/day of DPH for 11 months. Serum DPH was 1.6 mg/100 ml. Note the increased FAA in the transferrin zone.

Fig. 4. Chromatography of the serum of a 17-year-old patient with osteomyelitis. Symbols as in figure 1. Note the decreased FAA in the [illegible] zone.

Results

Table II shows the normal distribution of FAA in the chromatographies of young people. According to the results, the most important carrier protein is α_2 macroglobulin, the second transferrin and the third albumin. FAA is hardly found at all in the [illegible] zone.

In the DPH users the total serum FAA was reduced while protein binding increased. A slight fall in protein binding is noted in the α_2 macroglobulin zone.

A review of the chromatogram analyses for the other patients (table III, No. [illegible]) shows that, where the total serum FAA had been low (below 3 ng/ml) the percentage of binding to proteins was high. The

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Acta Haemat. [illegible] (1973)

The Prevalence of Australia Antigen and Antibody in Haemophilia

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Abstract. 15 haemophiliacs including one patient with von Willebrand's disease and 8 [illegible] transfused patients (7 homozygous sickle cell anaemia, 1 refractory anaemia in pregnancy) were examined by the immunoelectroosmophoresis (IOD) method for the prevalence of Australia antigen (Au(1)) and antibody among them. No Au(1) antigen or antibody was detected among the haemophiliacs. A [illegible] homozygous sickle cell anaemia had Au(1) antibody. Possible explanations for these findings are given.

Key Words

Australia antigen and antibody

Blood transfusion

Haemophilia

von Willebrand's disease

The introduction of cryoprecipitate [15] substantially simplified the management of classical haemophilia. The various modifications of the original procedure have made cryoprecipitate readily available in most countries. However, the discovery of Australia antigen (Au(1)) by Blumberg [2], its frequent association with viral hepatitis [3, 9], its presence in plasma, cryoprecipitate and other plasma fractions [16, 22] and its occurrence in patients with [illegible] disease and [illegible] of blood donors [7, 8, 12] introduced a new element of complexity in the management of haemophilia. In areas with low Au(1) incidence in the [illegible] population and among [illegible] blood donors, for example 0.1% in New York City donors [16], the existence of this antigen does not constitute a serious problem. Among Nigerian blood donors, however, the incidence of Au(1) is 6.0% [illegible] [illegible] prevalence constitutes a very serious problem.

Prior to January 1971, when routine screening of all blood donors for the Au(1) antigen and antibody was introduced here, it is certain that some [illegible] haemophiliacs [illegible] blood [illegible] the Au(1) [illegible] and became [illegible]

cidence of between 17 and 33% of anti-Au(1) antibody has been reported among haemophiliacs [19], we recently examined our patients for the prevalence of the Au(1) antigen and antibody among them. We also examined a matched group of other multiple transfused patients in order to establish the extent to which other factors in addition to the transfusion of probable Au(1)-containing blood affected our results.

Materials and Methods

Haemophilic patients. These were 14 patients who had previously been diagnosed as either haemophilia A or B (factor VIII or factor IX deficient) and one case of von Willebrand's disease who had been attending the Haematology and other Clinics in the University College Hospital, Ibadan [4-6]. Nine patients were originally examined and matched by age and socio-economic status with a control group (see below). Subsequently 6 other patients were included in the investigation but these were not matched with a control group.

The group comprised mild, moderate and severe haemophiliacs [10] aged between 2 and 22 years. Their mean requirement for transfusion of blood/blood fractions over a 3-year period was 22 U (range 5-60): red cells, fresh frozen plasma (FFP) and cryoprecipitate units inclusive. The various fractions used were usually derived from separate donors. Patients with high mean requirements were treated for surgical (including neurosurgical) complications of their disease state.

Other multiple transfused patients. This group was made up of 8 patients aged between 1 and 25 years, 7 of whom at the time of our investigation had been attending the Paediatrics Department Anaemia Clinic and some had received multiple blood transfusions. The eighth patient attended the antenatal clinic for refractory anaemia in pregnancy. Their mean blood transfusion requirement was 5 U (range 1-14).

Serum samples. During the preliminary tests, stored frozen plasma samples from the haemophiliacs were used. Subsequently, freshly collected samples from both the haemophiliacs and the multiple transfused patients were used. All samples were left to clot at room temperature before they were centrifuged at 3000 rpm for 10 min and the serum samples separated. These were set up on the day of collection by the immuno-electro-osmodiffusion method (IOD) of BEDARIDA *et al.* [1]. The remaining serum samples were frozen and preserved at -20 to -70 °C.

Results

Au(1) and anti-Au(1) in haemophiliacs. 14 haemophilic patients and one patient with von Willebrand's disease were tested for both the Au(1) and anti-Au(1). None of the samples gave positive results for either the antigen or antibody.

Table I. Summary of results of Australia antigen antibody and other tests on haemophiliacs and other matched multi-transfused patients

Haemophiliacs				Other patients			
patient No.	age years	factor VIII or IX defect	transfusions received units[1]	patient No.	age years	diagnosis	transfusions received units[1]
1	5[illegible]	VIII	6	1	6	HbS	1
2	22	VIII	31	2	25	refractory anaemia in pregnancy	14
3	6	VIII	13	3	7	HbS	7
4	3	VIII	43	4	2½	HbS	1
5	10	IX	3	5	9½	HbS	1
6	2	VIII	12				
7	4	VIII	60	6	3	HbS	2
8	½	VIII	5	7	1½	HbS	2
9	10	VIII	14	8	8	HbS	5

[1] Units of blood transfused in haemophiliacs included whole blood, packed cells, cryoprecipitate and fresh frozen plasma. In others, only whole blood and packed cells were used.

Au(1) antigen and antibody in the control group of multi-transfused patients. In the control group, one patient aged 2½ years who had received 100 ml of packed cells of Au(0) blood 5 months earlier, had anti-Au(1) (table I).

Discussion

The prevalence of [illegible] of Au(1) antigen among our blood donors [2[illegible]] is very high when compared with other published data, for instance, 0.1% among New York City volunteer donors [10]. It is therefore very likely that we had transfused some Au(1) positive blood and blood fractions into some at least of our patients before we introduced routine testing of blood donors for the Au(1) antigen and antibody in our hospital. All 1[illegible] haemophiliac and other patients whom we have examined, however, were negative both for Au(1) and the corresponding antibody, even though 14 received a mean total of 22 U of blood or blood fractions.

The results of low Au(1) antigen among our haemophilic patients are in agreement with those of others. Our results in respect of anti-Au(1) are, however, quite different. For instance, in environments with low Au(1) incidence both in the general population and among blood donors [16], anti-Au(1) has been reported in 29% of haemophiliacs (8 out of 28 patients), 28.6% (2 out of 7), 33% (7 out of 21), 13% (5 out of 39) and 17% (8 out of 48 patients) [7, 12, 18]. Our results cannot wholly be explained on the basis of our relatively small number of patients since the antigen is more than 60 times as frequent among our blood donors as among those in the populations where these other data were collected. We have used the same method by which other results were obtained. Moreover, it has been shown [17] that there is a 97-percent concordance between Au(1) results obtained by immunodiffusion and the more sensitive complement fixation methods.

Possible explanations for the higher prevalence of Au(1) among some healthy tropical population groups, lepromatous leprosy patients, leukaemic patients and children with Down's syndrome include the existence among such groups of widespread chronic anicteric hepatitis, genetic predisposition or defective immune mechanism with the result that they are more liable to infections [12]. We would include environmental factors in this list, for instance, exposure to mosquito bites [21]. None of our patients has evidence of any of the clinical states listed. But it is certain that at some time prior to this study, they had all been exposed to mosquito bites.

One possible explanation of our results could be that the total volume of blood and blood products which our patients have received is smaller than the volumes transfused into the haemophilic patients in other published series. It is difficult to comment on this point as relevant data are not available. It is, however, known that as little as 1 U of blood is adequate to infect the recipient and the antigen so acquired may persist for years. The results in our control series suggest that less than 1 U of blood can be infective. The 2½-year-old child with anti-Au(1) received 100 ml of Au(0) blood and 5 months later was found to carry anti-Au(1). The suggestion in this case is that she may not have acquired Au(1) to which she developed the antibody from the blood transfusion but probably from the ubiquitous insect (? mosquito) bite.

There is at present no evidence to suggest that our patients possess a more efficient immunological system which enables them to clear transfused Au(1) more readily than the apparently healthy blood donors, even

though it is known that adult Nigerians have higher levels of IgG and IgM than Europeans [13]. Further work in this area is receiving attention.

Acknowledgement. We are grateful to Profs. A.U. Antia and J.P. de V. Hendrickse, respective Heads of the Departments of Paediatrics and of Obstetrics and Gynaecology, for permission to study and report on their patients, to Prof. L. Luzzatto of the Haematology Department for his helpful comments and to Mr. E. Di[illegible] for technical assistance. Part of this work was supported by a grant from the Medical Research Council in Britain.

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Acta Haemat. 50: 299–304 (1973)

Blood Group and Tissue Mosaicism in a Natal Indian Woman

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Abstract. A normal 46, XX karyotype was demonstrated in an Indian woman who has 2 approximately equal populations of red cells and of leucocytes. She also has skin mosaicism. She is a normal female and has borne 4 healthy children. The ABO, Ss, Rh and Fy groups of her 2 red cell populations differed, but both were Le(a+b–) although she is a secretor of B and H substances. The relationship between this and the 7 examples of dispermy with blood group mosaicism reported in the literature is discussed briefly.

Key Words
Blood group mosaic
Dispermy
Karyotype
Mosaicism
Tissue mosaic

The condition of dispermy was first recognised in man in 1962 by Gartler *et al.* [8] in an XX/XY hermaphrodite who had blood group mosaicism. Since then 6 further cases have been recorded, 2 of them with additional skin mosaicism [1, 5, 11, 16]. Five were Caucasians [1–5, 7, 10, 11, 14] and one was a Negro American Caucasian [1, 16]. Some evidence of hermaphroditism and XX/XY karyotypes was present in 6 of the cases [1, 4, 5, 7–11, 14, 16], and one, a male propositus, was a mosaic for trisomy 21 [3]. Only the original case was orientated as a female, the 2 year old white girl of Gartler *et al.* [8, 9]. Dispermy is believed to be the result of separate fertilisation of one or more eggs or polar bodies by 2 sperm, but the exact nature of the female contribution, and when and how the products of fertilisation unite to develop into a single individual, is uncertain.

In this paper details are given of an eighth case of dispermy encountered in 1968 [13] when an Indian woman was found to have blood group and tissue mosaicism. She was a normal female with an XX karyotype

and had borne four normal healthy children. A preliminary account of her blood groups was included in the review by RACE and SANGER [15].

Case History

When first seen the Indian woman, Mrs. T R., who was a member of the Tamil sect of the Dravidian Indians, was 6 months pregnant with her fourth child. She was in good health and had come to the antenatal clinic for a routine examination. Mixed fields of agglutinated and unagglutinated red cells were seen in tests with anti-D. No history of a recent blood transfusion or bone marrow grafting was elicited and the ABO group of her fourth child at birth excluded the possibility of fetomaternal transplacental haemorrhage (fig. 1). Dispermic mosaicism, as opposed to twin chimerism, was suspected when Mrs. T R.'s mother stated that there had been no twin or unexplained tissue at the birth of her daughter. Blood group tests on several occasions during the following 6 years, when Mrs. T R. was not pregnant, confirmed the persistence of the 2 populations of red cells.

Results

Red cell studies. In addition to the unexpected mixed agglutination with anti-D, further evidence of the presence of more than one type of red cell was obtained when Mrs. T R.'s cells were grouped with anti-A, anti-C, anti-S and anti-Fy^b. In each test approximately 50% of the cells were agglutinated. Two distinct red cell populations were separated by using anti-A serum [2], and on a later occasion with incomplete anti-D serum followed by antiglobulin reagent. The groups of the separated cells were as follows: (1) A_1B, MNSs, P_2, Tj(a+), cde cde, Lu(a−b+), kk Kp(a−b+) Js(a−), Le(a+b−), Fy(a−b+), Jk(a+b−), Xg(a+), Do(a)+, Co(a+b−) Vel+, I+. (2) B, MsNs, P_2, Tj(a+), CDe cde, Lu(a−b+), kk Kp(a−b+) Js(a−), Le(a+b−), Fy(a+b−) Jk(a+b−) Xg(a+), Do(a+) Co(a+b−), Vel+, I+. Ashby counts [12] revealed 45% A_1B D-negative cells and 55% B D positive cells, supporting the original 50:50 estimate.

Lewis antigens and secretions. Figure 1 shows that both the separated red cell populations were Le(a+b−). As Mrs. T R. secreted B and H substances in her saliva, RACE and SANGER [15] pointed out that in these respects she is very similar to the Detro t example of d spermy [1, 16] who secreted A and H substances although all his red cells were Le(a+b−). BEATTIE *et al.* [1] deduced that the tissue formed from the minor (10%) genetic product at fertilization in their case possessed the genes

sese Lele and was capable of forming enough Le^{a} substance to coat the red cells of both populations. Since Mrs. T. R.'s father was dead and her mother was not tested for secretion it was impossible to decide whether the tissue producing her group B D-positive cells was genotypically Sese Lele and her Lewis groups really Le(a–b+), as suggested by BEATTIE *et al.* [1] for the major (90%) A_1O component in the Detroit example, or was Sese lele with cells Le(a–b–).

Two of Mrs. T. R.'s children were group O (fig. 1), strongly supporting the view that her reproductive cells and the tissue that was producing her group B, D-positive red cells were derived from the same original component at fertilization. If her A_1B, D negative red cell-producing tissue had been associated, she would have had only A or B children. It was unfortunately impossible to assess with certainty the maternal and paternal Lewis and secretor gene contribution in this case, but RACE and SANGER [15] suggested that Mrs. T. R.'s mother was very probably B Sese, and her father had contributed A_1, se and O, Se sperm.

Leucocytes. HL-A grouping showed that there was an excess number of HL-A antigens present in Mrs. T. R.'s peripheral circulation. This is consistent with the existence of 2 different leucocyte populations. The antigens found were at the first (LA) locus, HL-A1, HL-A10 and W28 and at the second (Four) locus HL-A8, HL-A13 (doubtful) and W5.

In all the reactions that were considered positive only half the number of leucocytes present reacted with each antiserum. This was in accordance with the 50:50 distribution estimated for the 2 populations of red cells. The Turin example of dispermy [4, 10], in which the lymphocytes were reported to differ by 2 HL-A haplotypes, was said to be genotype ac and bd in approximately equal proportions.

Skin mosaicism. The skin of both of Mrs. T. R.'s thighs was completely covered with fine, intimately interwoven, striations of light and dark-brown pigmentation. The skin of her trunk was said to be marked in the same manner, but only the lighter of the 2 shades of pigmentation was observed on the head, arms and shoulders. This type of skin mosaicism is quite unlike the case reported by ZUELZER *et al.* [1, 16] or that of COREY *et al.* [5, 11] in which skin 'patchiness' was described. The former (the Detroit example) had multiple small discrete areas of darker skin on the face, with fairly symmetrical wedges and bands extending in a lateral direction from both the anterior and posterior midline on the trunk, buttocks and thighs. The latter (the Vancouver example) had large mottled areas, most conspicuous on the abdomen where they formed patches of

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Acta Haemat. 57: 305–314 (1977)

Acquired Factor IX Deficiency

A Report of Two Cases

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Abstract. Coagulation studies in 2 patients revealed decreased factor IX activity. Family history of factor IX deficiency or bleeding disorder was not present and the past history of the patients did not disclose any evidence of congenital hemorrhagic disorder. They did not require any plasma or plasma product for the control of their bleeding diathesis and decreased factor IX activity returned to normal in a relatively short period of time. Factor IX deficiency was due to a circulating anticoagulant. With corticosteroid treatment, marked clinical improvement occurred in the second patient with some improvement of her coagulation status, but it did not seem to effect the circulating anticoagulant level in the first patient.

Key Words
Anticoagulants
Bleeding disorders
Blood coagulation
Factor IX deficiency

Acquired factor VIII deficiency has been reported in previously normal persons [1–5], in patients with collagen diseases, in patients treated with penicillin [1, 6, 7], following electric shock [8] and in women following parturition [1, 9]. Von Willebrand's syndrome [10–12] and factor IX deficiency [13–17] may also be acquired. We report here transient factor IX deficiency in a 14-year-old boy and in a 31-year-old female.

Case Reports

Case 1. N.C. (H.U.H. No. 197538), a 14-year-old male was well until 12 days before being seen at the Hacettepe University Hospital [illegible] Medical Center when he developed pain in the ankles and some bleeding of 3 days duration. There was no family history of a bleeding tendency. The patient was [illegible] at a

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Acta Haemat 50 305–314 (1973)

Acquired Factor IX Deficiency

A Report of Two Cases

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Abstract Coagulation studies in 2 patients revealed decreased factor IX activity Family history of factor IX deficiency or bleeding disorder was not present and the past history of the patients did not disclose any evidence of congenital hemorrhagic disorder They did not require any plasma or plasma product for the control of their bleeding diathesis and decreased factor IX activity returned to normal in a relatively short period of time Factor IX deficiency was due to a circulating anticoagulant With corticosteroid treatment marked clinical improvement occurred in the second patient with some improvement of her coagulation studies but it did not seem to effect the circulating anticoagulant level in the first patient

Key Words
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Acquired factor VIII deficiency has been reported in previously normal persons [1–5] in patients with collagen diseases, in patients treated with penicillin [1, 6 7] following electric shock [8] and in women following parturition [1, 9] Von Willebrand s syndrome [10–12] and factor IX deficiency [13–17] may also be acquired We report here transient factor IX deficiency in a 14 year-old boy and in a 31 year-old female

Case Reports

Case 1 S C. (HUH No 199336) a 14 year-old male was well until 10 days before being seen at the Hacettepe University Hospital Children s Medical Center, when he developed pain in the ankles and nose bleeding of 3 days duration There was no family history of a bleeding tendency The patient was circumcized at 4

years of age and had teeth extracted at 8 years of age without excessive bleeding One brief episode of epistaxis occurred in early childhood it did not require any treatment and did not recur Physical examination showed a well-developed pale boy whose pulse was 108 per minute and blood pressure 120 80 mm Hg A grade $^{2}/_{6}$ systolic murmur was heard over the cardiac apex the liver and spleen were not palpable and there were no ecchymoses or petechiae

The hemoglobin (Hb) was 9 8 g/100 ml hematocrit (Hct) 29% WBC 10 300/ mm³, with a differential count of 70% granulocytes 1% bands 2% monocytes and 27% lymphocytes and there was 1 normoblast per 100 WBC Platelets were adequate on the peripheral smear and the red cells were normocytic, normochromic *The sedimentation rate was 45 mm/h (Westergren) ASO titer 500 Todd units* CRP, Coomb s antiglobulin test and LE phenomenon were all negative Total serum protein was 7 4 g/100 ml and electrophoresis showed albumin 41% α_1 1 8% α_2 19 4% β 19 2% and γ globulin 18% Immunoelectrophoresis was normal Urinalysis showed 1+ proteinuria and microscopic hematuria EKG disclosed slightly prolonged PR interval X rays of the chest and extremities were normal and a throat culture was negative Coagulation studies are reported separately

The patient received corticosteroids, penicillin and iron Three weeks later his Hb rose to 12 3 g/100 ml Hct to 37% and his platelet count was 256 000 mm³ Intermittent nose bleeds occurred for the next 2 months When last seen in April 1971 he was symptom free

Case 2 M A (HUH No 208806) a 31 year old housewife was admitted to Hacettepe University Hospital on October 7, 1970 because of shortness of breath She had been in good health until 18 months before admission when a cough and hoarseness developed during the early months of her first pregnancy, lasting 2 weeks, similar symptoms recurred 4 months later again lasting 2 weeks 13 months before admission she began having frequent nose bleeds and spontaneous ecchymoses, and 1 week before admission shortness of breath cough hoarseness and high fever occurred On admission the physical examination showed BP 100/70 mm Hg pulse 140 per minute respiration 24 per minute and temperature 37 °C. The skin and mucous membranes were pale and there were large ecchymoses over the buttocks The throat was red and her voice was hoarse The liver was palpable 8 cm below the right costal margin the spleen was not palpable and the remainder of the examination was noncontributory

The Hb was 8 7 g/100 ml Hct 30% WBC 9 000 mm³ with a differential of 3% myelocytes 7% metamyelocytes 5% bands 20% polymorphnuclear (PMN) leukocytes 8% monocytes and 57% lymphocytes There were 18 normoblasts per 100 WBC On the peripheral smear the red cells showed moderate anisocytosis and polychromasia and the platelets were decreased Total serum protein was 6 5 g albumin 3 0 g globulin 3 5 g bilirubin 0 8 mg cholesterol 80 mg/100 ml alkaline phosphate 9 8 Bodansky units SGOT 100 Karmen units and BSP retention was 22% after 45 min Serum electrolytes blood sugar urea nitrogen and SGPT were normal Serum iron was 199 μg and total iron binding capacity 492 μg/100 ml Serum protein electrophoresis revealed albumin 34% α_1 4% α_2 24% β 7% and γ globulin 31% Serum antinuclear factor (ANF) LE cell phenomenon cryoglobulin cryofibrinogen and direct and indirect Coombs tests were negative A bone marrow aspiration showed hypercellularity with erythroid hyperplasia and lymphocytosis Direct

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Table I. Pertinent coagulation findings

Tests[1]	Case 1			Case 2			
	8/25/70	8/27/70	9/7/70	11/2/70	11/4/70	11/27/70	6/1/71
Clotting time, min (<12)	4 5	4	7 5	4	7	5	9
Bleeding time, min (<6)	1 5	ND[2]	2	5	17	20<	16
Retraction (2–4+)	2+	2+	2+	0	0	0	ND
Petechiometer (–)	–	ND	–	ND	ND	ND	ND
Quick prothrombin time, sec (<12)	14	14	13	15	17	18	17
PTT, sec (60–120)	142	154	165	198	178	200	118
Factor II, % (60–200)	ND	79	113	ND	ND	67	60
Factor V, % (60–200)	ND	86	110	ND	ND	82	160
Factor VII–X, % (60–200)	ND	60	83	ND	ND	50	85
Factor X, % (60–200)	ND	62	109	ND	ND	59	119
Fibrinogen, mg% (200–400)	ND	397	ND	ND	ND	ND	ND
Factor XIII (– to 1+)	ND	1+	1+	ND	ND	ND	ND
Platelets (150–400,000 mm³)	ND	278,000	265,000	decreased	208,000	66,000	132,000
Platelet aggregation, sec (<18)	ND	11	ND	ND	ND	ND	ND

[1] Numbers in parentheses indicate our normal values
[2] ND = not done

Table II. TGT studies, sec[1]

	NS + N ad pl[2]	NS + Pt ad pl	Pt S + N ad pl	Pt S + Pt ad pl
Case 1				
8/25/70	9 3	9 2	13 1	11
8/27/70	8 9	8 2	13 7	11 6
9/9/70	9 5	9 3	13 9	11 4
1/13/71	9 1	9 2	9 5	9 2
Case 2				
11/2/70	10	9 8	22	24 2
11/4/70	8 5	8 4	22	23 7
11/27/70	8 7	8 2	16 8	17 2
6/1/71	8 6	8 4	9 2	8 4

[1] Only minimal substrate clotting times were recorded
[2] NS = normal serum, Pt S = patient serum, N ad pl = normal adsorbed plasma, Pt ad pl = patient adsorbed plasma

Table III PTT studies of case 1, sec

	Before incubation	After 0 5 h incubation at 37°C
Normal plasma	110	
Patient's plasma	165	
½ normal plasma + ½ VB[1]	114	113
½ normal plasma + ½ patient plasma	114	162
½ patient plasma + ½ VB	224	232

[1] VB = Veronal buffer

Table IV TGT studies, sec Anticoagulant effect of the patient's serum before and after incubation for 0 5 h at 37°C[1]

	Before incubation	After 0 5 h incubation at 37°C
Case 1 (Sept 9, 1970 serum)		
NS[2]	9 5	
Pt S	13 9	
½ NS + ½ VB	10 9	10 6
½ NS + ½ Pt S	10 9	17
½ Pt S + ½ VB	17 2	32
Case 2 (June 1, 1971 serum)		
NS	8 6	
Pt S	9 2	
½ Pt S + ½ Pt S (obtained Nov 27, 1970)[3]	14 0	16 2
½ Pt S + ½ VB	9 6	9 5

[1] Normal adsorbed plasma was used and only minimal substrate clotting times are recorded

[2] Abbreviations as in tables II and III

[3] Kept frozen

levels in both 11% in the first patient (Sept 30, 1970), and 7 8% in the second patient (Nov 2, 1970 sample) Circulating anticoagulant(s) against factor IX was sought by mixing the patients sera and normal serum (table III, IV) The first patient's PTT findings suggested and the TGT results confirmed a circulating anticoagulant against factor IX, which disappeared by January 13, 1971 No anticoagulant could be demonstrated in the November 4, 1970 sample of the second patient's serum However, when the sera obtained on November 27, 1970 and June 1, 1971 were mixed, the TGT became abnormal (table IV) suggesting the presence of an anticoagulant in the November 27, 1970 sample

Comment

Acquired hemophilia A has been reported [1–5, 7, 8, 27–29] more often than acquired hemophilia B [1, 13–17] Both of our patients are examples of the latter The first patient, an adolescent boy with a negative family and past history of bleeding tendency, had a clinical picture consistent with rheumatic fever in which epistaxis is common Because of the unusual severity of his nose bleedings further investigation was undertaken and the transient factor IX deficiency was found In his case the factor IX deficiency was due to a circulating anticoagulant In hereditary hemophilias, circulating anticoagulants occur most often following repeated transfusions [30, 31] Patients with collagen diseases also may develop circulating anticoagulants against factor VIII [1, 10–12, 28] However, we found in the literature only 2 cases of acquired factor IX deficiency due to circulating anticoagulants [14, 16] It was not shown in 3 [13, 15, 32] and was most likely in one case of acquired factor IX deficiency [17] There seems to be no good explanation for the sudden development of an anticoagulant against either factor VIII or IX but it is interesting that our patient had an illness consistent with rheumatic fever in which 'autoimmune' phenomena are known to occur [33]

Although von Willebrand's syndrome and rarely hemophilia A [34–37] and B [38, 39] do occur in females, in our second patient the factor IX deficiency was transient as is in all reported cases of acquired hemophilia B There was only suggestive evidence of a circulating anticoagulant in one sample of her sera, all other samples showed the factor IX deficiency only No satisfactory explanation for this was found Apparently

in association with the factor IX deficiency, her clinical picture (characterized by fever, inflammation of the upper respiratory tract, hyperglobu linemia and perhaps, thrombocytopenia) was reminiscent of systemic lu pus erythematosus a disease in which acquired hemophilia A [1–3], hemophilia B [17] and von Willebrand's syndrome [10] have been report ed ANF antibodies and LE phenomenon however, were absent Acquired factor IX deficiency has been reported following viral infections, such as viral hepatitis [14] and although the clinical picture here was not suggestive of a viral infection, the possibility of a concomitant infection cannot be excluded without specific studies which we were not able to carry out The role of pregnancy is also unclear, although acquired hemophilia A has been reported [1, 9] following parturition

Thrombocytopenia and prolonged bleeding time may occur in myeloid metaplasia [40] which our patient had, but it would be difficult to explain the factor IX deficiency on this basis alone, because of the transient nature of the latter Factor IX deficiency can occur in disseminated intravascular coagulation [41], but this too is unlikely in the case under discussion since there was no evidence of factor VIII or other clotting factor deficiencies when the factor IX level was low

To date, all reported cases of acquired factor IX deficiency have re covered and this was true in our patients The first patient with acquired factor IX deficiency [14] was treated with ACTH and the beneficial effect of corticosteroids was stressed in 2 other patients [15, 32] Marked clini cal improvement occurred in our second case with steroid therapy, but the value of this treatment in our first patient is very questionable since the factor IX deficiency disappeared several months after the steroids were discontinued Clot promoting effect of corticosteroids is well known [42, 43] and their effect on factor IX level in a patient with Sheehan's syndrome has recently been reported [32] But their efficacy in acquired factor VIII and IX deficiency has been questioned by others [1, 4, 7, 9, 28] Acquired factor IX deficiency is a rare disorder Perhaps this is more apparent than real, as the discovery of 2 cases in a short time may indi cate Investigation of 'minor' bleeding tendencies may lead to discovery of more cases

Acknowledgements We are grateful to Drs WILLIAM BELL, M B STEVENSON, A E MURPHY V A MCKUSICK all from Johns Hopkins Hospital for ANF test and for helpful suggestions, to Mr A AY and Mrs F ACIR for valuable laboratory assistance and to Mrs B EROZAN for editorial help

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Acta Haemat. 50 315-320 (1973)

Haemoglobin D Punjab in a Cuban Family and its Interaction with Haemoglobin S

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Abstract A Cuban child carried 2 abnormal slow moving haemoglobins and suffered from severe haemolytic anaemia Haematological studies of the family and chemical analysis of the abnormal haemoglobins indicated that he was a case of sickle-cell haemoglobin D disease, which is produced by the interaction between Hb S and Hb D Punjab This is the first case of Hb D Punjab found in Cuba The clinical picture of the patient is discussed

Key Words
Haemoglobinopathies
Hb D Punjab in Cuba
Hb S
Sickle-cell Hb D disease

The classification haemoglobin D (Hb D) comprises the haemoglobin variants which are indistinguishable from Hb S in starch gel electrophoresis at pH 8 6 and in chromatography, but do not show the characteristic insolubility of Hb S in the reduced form, and thus do not have the ability to cause sickling [6] A number of such haemoglobin variants structurally different have been described [4] Among them Hb D Los Angeles, or Hb D Punjab is the most common since it is widely distributed in the Sikhs of Punjab [8] Sporadic cases have also been found in European countries as well as in the United States, and recently one family has been found in Thailand [4, 13] It has been suggested that this haemoglobin mutant has been exported from India, specially to countries which have had economic and political contacts with this country, although the possibility of independent mutations cannot be excluded

The interaction between Hb D Punjab and Hb S is known as 'sickle-cell haemoglobin D disease', and in fact this Hb D was first described by ITANO [7] in a patient suffering from this disease Many cases of sickle-cell haemoglobin D disease have been reported, although only in a

few of them Hb D was chemically identified The main feature of this disease is that of an haemolytic anaemia resembling sickle cell anaemia in the mildest form, but the symptomatology of the cases so far reported presents a wide degree of heterogeneity We want to report a case of Hb D Punjab found in a Cuban family and its interaction with Hb S

Case History

D C F was admitted at the William Soler Hospital in Havana in January 1968 when he was 3 months old He was first noted pale by his mother at the age of 1 month and diagnosis of anaemia was made in a Hospital of his locality At the time of his admission he was remarkably pale a slight jaundice was observed the liver and the spleen were 6 and 11 cm below the right and left costal margins respectively There was no history of anaemia in either parents The father was a mestizo and the mother was a white descendant of immigrants from Spain

Haemoglobin 4 5 g%. Leucocytes 10,921 Differential count neutrophils 35%. basophils 2% metamyelocytes 4% myelocytes 2% monocytes 5% lymphocytes 52% Peripheral blood showed marked hypochromia anisocytosis poikilocytosis drepanocytes polychromatophilia Howell Jolly bodies target cells basophilic stippling and normoblasts Serum iron 174 μg% Bilirubin total 1.2 mg% direct 0 155% Bone marrow showed marked erythropoietic activity with megaloblastic changes and the iron staining was intensely positive The electrophoretic pattern of his hemolyzate was that of an homozygote for Hb S with 12% foetal haemoglobin

During his stay in the Hospital he had persistent thrombocytopenia and very low haemoglobin values, for which several transfusions were required and several months later splenectomy was decided after which a postoperatory *Klebsiella aerobacter* infection developed In the meantime the parents were studied (see Results and Discussion) and the previous diagnosis of sickle cell disease was changed to that of sickle cell haemoglobin D disease

The propositus has been followed at the outpatient clinic His physical growth was acceptable, but he showed slight mental retardation After splenectomy he has had several infections at the upper respiratory tract kidneys skin lungs and a severe bacterial meningoencephalitis He was transfused during some of these infection episodes but he did not need any transfusion in between The haemoglobin fluctuated between 5 and 7 g% and the reticulocyte count between 6 and 18% with many normoblasts in the peripheral blood The liver remained 12 cm below the costal margin Recently he was admitted to the Hospital for bone pains, but painful crises has been noticed seldom so far

Methods

Routine haematological data were obtained by standard methods [3] Starch gel electrophoresis was carried out at pH 8 6 using the discontinuous buffer of POULIK

Table I Haematological data of the family

	Hb g%	PCV °₀	RBC $\times 10^6$/mm³	MCV μ^3	MCH $\mu\mu g$	MCHC °₀	Reticu locytes %	Serum iron μg%	HbF %	Solu bility test
Father	13 4	42	4 60	91	29	31 8	1.2	95	0 7	positive
Mother	12 5	40	4 30	90	29	31 2	1 8	84	0 5	negative
Propositus	6 9	24	2 05	120	34 5	28 7	12 1	174	12 2	positive

[9] The *solubility* test was done according to the method of ITANO as modified by GOLDBERG [5] Foetal haemoglobin was quantitated by the alkali denaturation method of BETKE *et al* [2] Chromatographic purification of the abnormal haemoglobin was obtained on diethylaminoethyl (DEAE) cellulose, using for the elution a linear ionic gradient of tris phosphate buffer at pH 8.5 [4] Tryptic digestion of the heat-denatured purified abnormal haemoglobin was performed at 38 °C, and the peptide mixture obtained analyzed by the fingerprinting technique of INGRAM as modified by BAGLIONI [1] The standard staining techniques for the identification of peptide spots and for specific amino acids were used Peptides to be eluted for further analysis were localyzed by staining the peptide map in 0 02% ninhydrin solution in acetone, containing 5% of a pH 6 4 buffer (pyridine acetic acid water = 25 1 224), and heating at 60 °C for 20 min The peptides were eluted with 6 N HCl and hydrolyzed at 107 °C for 24 h *in vacuo* After evaporation the sample was dissolved in water and the amino analysis performed in a Beckmann amino acid analyzer model 120B

Results and Discussion

Clinical manifestations and haematological data of the propositus firstly suggested either a sickle-cell disease or a sickle-cell β-thalassaemia Due to the high frequency of the S gene in our country the most likely diagnosis was that of sickle-cell anaemia, as indicated also by the *electrophoretic pattern of the parents* which was typical of the sickle-cell trait However, the solubility test of the father was positive, whereas the mother's haemolyzate did not show any insoluble haemoglobin It was thus concluded that the propositus was a carrier of both Hb S and another haemoglobin with the *same electrophoretic* mobility The haematological data of the family are shown in table I

Interactions between Hb S and other haemoglobins have been described each giving different manifestations Thus, interactions between

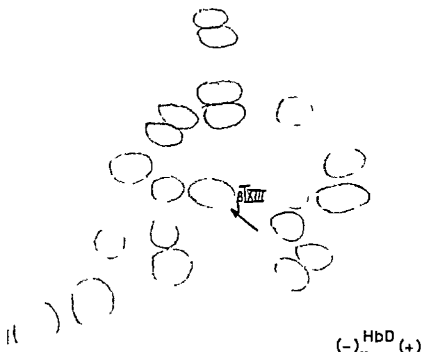


Fig 1 Fingerprint of the Hb D Punjab The abnormal peptide is shown by arrow

Hb S and Hb C, Hb G, Hb D Ibadan have been found, the most serious from a clinical point of view being that between Hb S and Hb D Punjab This entity is similar to sickle-cell disease, but shows a great degree of heterogeneity as far as clinical consequences are concerned The clinical picture of the cases so far reported varies from an asymptomatic state to that of a severe anaemia [10–12]. Unfortunately, only in a few cases the Hb D was chemically identified

In order to compare our case with the others previously reported, being sure that our haemoglobin was Hb D Punjab, the abnormal haemoglobin was purified from the mother's haemolyzate and analyzed as described under Methods The fingerprint of this haemoglobin, shown in figure 1 is that of Hb D Punjab in which an aspartic acid is substituted for an asparagine in position 121 of the β-chain This was confirmed by the amino acid analysis of the abnormal peptide It was thus concluded that our propositus was a case of sickle-cell-Hb D disease The patient presented an evolution particularly serious mainly during the first year of life Levels of haemoglobin lower than 5 g% were maintained during

this period, which made it necessary frequent transfusions and kept the patient in a state of continous danger

The other cases reported, although characterized by great variability, showed all the feature of painful crises which dominated the clinical picture Our case is not characterized by such crises but he resembles to the patient reported by STURGEON *et al* [12], who also required splenectomy at 1 year of age It is possible that the great splenomegaly was the cause that made worse the clinical picture and the anaemia during the first month of life After splenectomy, although haemoglobin levels only raised to approximately 7 g%, the patient required blood transfusions only during severe infections which have been frequent, as described in the Case History It is well known that this is not an unusual finding, for infections are common in haematological patients precociously splenectomized We can say that our patient had the clinical course of a congenital serious haemolytic anaemia, which could have been easily confused with sickle cell anaemia in the most severe form and of precocious debut

Acknowledgments We are grateful to Dr E DE LA TORRE for his advice and encouragement The amino acid analysis was performed by Prof ROMANO ZITO Istituto Regina Elena, Rome (Italy)

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Acta Haemat. 50 321–328 (1973)

The Value of Laparotomy and Splenectomy in the Staging of 56 Patients with Hodgkin's Disease

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Abstract The results of laparotomy and splenectomy in the staging of 56 patients with Hodgkin's disease are presented Negative or dubious lymphangiograms were inaccurate in 37% of the patients who had abdominal lymph node involvement Nonpalpable spleens were affected by Hodgkin's disease in 39% of the cases Palpable hepatomegaly is not a good sign for predicting liver involvement, nor are BSP or alkaline phosphatase determinations In 55% of the patients with involved spleen the liver was still free of disease The prelaparotomy clinical stage had to be changed in 43% of the cases as a consequence of the postlaparotomy results, permitting a more accurate therapeutic approach

Key Words
Hodgkin's disease
Laparotomy in Hodgkin's disease
Splenectomy in Hodgkin's disease
Staging in Hodgkin's disease

Since Easson and Russell [2], Kaplan [5, 6], and Peters [10] published their early results on radiotherapy in Hodgkin's disease, the possibility of 'cure' has been suggested Kaplan's experiments [5, 6] clearly demonstrated the existence of a tumoricidal level in the radiotherapy of Hodgkin's disease, tumor doses of 4,000 rads could erradicate the disease in the treated region with less than a 4-percent relapse rate

Nevertheless, radiotherapy of affected regions and prophylactic irradiation of areas suspected of occult involvement did not prevent early relapse on some patients who suffered an unknown or undiagnosed degree of disease involvement in the abdomen Despite lymphangiography, the abdominal cavity was still the most difficult body region as far as evaluation of the extent of Hodgkin's disease was concerned Laparotomy

seemed the logical procedure to be taken in order to achieve a correct staging More recently, GLATSTEIN *et al* [3] published their first results about the value of laparotomy and splenectomy in the staging of Hodgkin's disease Since then other reports [1, 4, 8, 9, 11, 12] have consistently confirmed their findings

Patients and Methods

This report contains the results of 56 patients with confirmed Hodgkin s disease who underwent laparotomy and splenectomy for the staging The study which lasted from January 1971 to June 1972 was a joint effort carried out by 2 hematological centers in Barcelona Escuela de Hematología Farreras Valentí' (31 patients) and Departamento de Hematología de la Ciudad Sanitaria 'Francisco Franco' (25 patients)

All patients, whose diagnosis of Hodgkin's disease was previously established by biopsy, were subjected to the following protocol (1) meticulous case history and physical examination, (2) chest X ray and whole lung tomography, (3) routine blood tests, bone marrow aspiration, plasma protein electrophoresis routine urinalysis, (4) liver functions tests – SGOT, SGPT, alkaline phosphatase (AP) and BSP retention, (5) bilateral lower extremity lymphangiogram, and (6) a number of patients were subjected to liver and spleen scans

Furthermore in selected patients, whose clinical signs and symptoms so warranted skeletal X ray studies, pyelograms, peritoneoscopy and/or liver needle biopsy were performed

Patients with evidence of disseminated disease (stage IV) and with any medical contraindications to elective abdominal surgery were excluded from the study

The modified classification recommended by the Nomenclature Committee at the Rye Conference was utilized Table I shows the histological classification of the 56 cases Note the predominance of nodular sclerosis in females and of mixed cellularity in males The study included a majority of patients in stages II and III (49 of 56) with an equal distribution of A and B cases The youngest patient was 15 and the oldest 64

Clinical criteria for determining abdominal spread of Hodgkin's disease included (1) 'abnormal' lymphangiogram, considered abnormal only when enlarged nodes containing filling defects were present, evidence of small filling defects in otherwise normal sized nodes was regarded as equivocal but clinically nondiagnostic, (2) palpable splenomegaly A smooth palpable hepatomegaly was not clinically considered as demonstrating liver involvement nor were abnormal BSP or AP values

In all cases the surgical procedure was performed by the same team in each center and consisted of the following steps (1) splenectomy, including removal of the splenic hilar region, (2) biopsy of the suspect periaortic and/or iliac lymph nodes when the preoperative lymphangiogram proved positive or equivocal, (3) exposure and exploration of the left periaortic nodes from the 12th thoracic to the 2nd lumbar vertebra and sampling of this area, (4) exploration of the porta hepatis, (5) exploration of the entire visible and palpable surface of the liver and sampling of two

Table I Histological classification of the 56 cases

	Male	Female	Total
Lymphocyte predominance	3	–	3
Nodular sclerosis	5	14	19
Mixed cellularity	20	13	33
Lymphocyte depletion	–	1	1
Total	28	28	56

wedge biopsies, one from each lobe, when no gross pathological finding was detect ed and (6) bilateral oopboropexy in young women Tantalium clips were left as ref erences when nodes were taken out and as ovarian markers after oophoropexy

Results

Several important correlations between prelaparotomy and postlaparotomy findings have been studied

Correlation of lymphangiogram and abdominal nodes (table II) In about 37% of the cases with a negative or dubious lymphangiogram, laparotomy discovered abdominal lymph node involvement. Most of them were located above L2 in the left periaortic region not usually filled by lymphangiography In contrast, in 28% (7 out of 25 cases) with a positive lymphangiogram, node involvement could not be confirmed by laparotomy

Correlation of palpable splenomegaly and spleen involvement (table III) Of 13 cases with palpable splenomegaly, 3 had no spleen involvement Thus, palpable splenomegaly is not always an indication of spleen involvement. On the other hand, nonpalpable spleens can be affected with Hodgkin's disease, as shown by this study, where 17 out of 43 (39%) were abnormal

Correlation of hepatomegaly and liver involvement (table IV) In 15 out of 21 patients the liver was palpable but not involved, indicating that a palpable liver is not a reliable clinical sign of hepatic involvement. On the contrary, a nonpalpable liver was involved in a small percentage of the cases (7%)

Correlation of elevated BSP retention and/or AP and liver involvement (table V) The liver was uninvolved in 4 out of 11 cases with abnormal BSP retention and/or increased AP, while in 5 of 38 patients with normal results it was affected

Table II Correlation of lymphangiogram and abdominal nodes

Lymphangiogram	Total	Abdominal nodes	
		uninvolved	involved
Negative	17	11	6 } (37%)
Equivocal	10	6	4 }
Positive	25	7 (28%)	18

Table III Correlation of palpable splenomegaly and spleen involvement

Spleen	Total	Spleen	
		uninvolved	involved
Palpable	13	3	10
Not palpable	43	26	17

Table IV Correlation of hepatomegaly and liver involvement

Liver	Total	Liver	
		uninvolved	involved
Palpable	21	15	6
Not palpable	35	29	6

Table V Correlation of elevated BSP and/or AP and liver involvement

BSP and/or AP	Total	Liver	
		uninvolved	involved
Abnormal	11	4	7
Normal	38	33	5

Correlation of spleen scan and spleen involvement (table VI) There is a poor correlation between a normal spleen scan and spleen involvement, 9 out of 15 patients (60%) had a diseased spleen but a normal scan The correlation improves when the spleen scan indicates pathological findings

Table VI Correlation of spleen scan and spleen involvement

Spleen scan	Total	Spleen uninvolved	Spleen involved
Abnormal	4	1	3
Normal	15	6	9

Table VII Correlation of liver scan and liver involvement

Liver scan	Total	Liver uninvolved	Liver involved
Abnormal	8	5	3
Normal	13	9	4

Table VIII Correlation of spleen involvement and liver involvement

Spleen	Total	Liver uninvolved	Liver involved
Involved	27	15	12
Uninvolved	29	29	0

Table IX Reclassification of stage 24/56 (43%)

Stage advanced	16
Stage reduced	8
Stage unchanged	32
Total	56

Correlation of liver scan and liver involvement (table VII) The liver scan is more reliable when it shows normal findings, 9 out of 13 normal liver scans had the liver uninvolved (70%) However, from 8 abnormal scans, the liver proved disease free in 5

Correlation of spleen involvement and liver involvement (table VIII)
In 12 out of the 27 cases with involved spleen, involvement of the liver was also present, but in no case where the spleen was normal, was the liver affected

Table IX summarizes the number of patients who required a postlaparotomy stage reclassification in 16 cases the stage was advanced, in 8 cases was reduced and in 32 cases remained unchanged Thus, 24 out of 56 patients (43%) needed restaging

Complications
One death occurred among the cases, a IIIB stage patient who died a month after laparotomy due to necrotizing enteritis One patient had to be relaparotomized one year after the original exploration because of intestinal obstruction and has had no further complications Two cases presented high fever a few days after laparotomy, one of them due to pneumonia A few patients presented wound infections of short duration It is surprising how well the great majority of patients with confirmed Hodgkin's disease tolerated and recovered from a major surgical procedure

Discussion

This report confirms previous publications on the value of laparotomy and splenectomy in the staging of Hodgkin's disease [1, 4, 8, 9, 11, 12] Rosenberg [12] found that approximately 25% of spleens, unsuspected as involved clinically turned out to have Hodgkin's disease when studied carefully by the pathologist Conversely, approximately 50% of spleens judged to be clinically enlarged did not contain Hodgkin's disease when examined histopathologically Our results are somewhat different, showing a better correlation between palpable and affected spleens (10 spleens affected out of 13 palpable splenomegalies), but a poorer one between nonpalpable and normal spleens (39% of nonpalpable spleens were affected) Possibly Rosenberg's criteria of 'clinically enlarged spleen' included radiologically enlarged spleens The survey revealed that 4 cases with a spleen less than 200 g had spleen involvement In 2 cases there was only one pathological node visible macroscopically with no other evidence of disease in the abdominal cavity Another case had only one pathological node in the splenic hilus and no other abdominal finding

The correlation of involvement of the liver and spleen is similar to that of the Stanford group [12] No examples of involvement of the liver without concomitant involvement of the spleen were found On the contrary, of 27 cases with involved spleen, 15 cases (55%) had normal livers In a previous study by GLATSTEIN *et al* [3] only 25% of cases with spleen involvement had a normal liver Our results are more in keeping with the experience of AISENBERG *et al* [1] who emphasizes the important role of the spleen and splenic hilar nodes as being possibly the first intraabdominal regions to be affected when Hodgkin's disease begins at the supradiaphragmatic level

BSP retention and AP determination are very poor in predicting the pathological involvement of the liver Likewise, spleen and liver scans are not usually very helpful in predicting involvement of these organs Only a normal liver scan can occasionally be useful The above results are also in agreement with other publications [7, 13]

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Acta Haemat 50 329–339 (1973)

Erythrodermia, Mycosis fungoides, Skin Reticulosis – *Autonomous Disorders of the Monocytopoietic Macrophage System?*[1]

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Abstract Detailed studies of monocytopoiesis and of blood monocytes were carried out in patients with erythrodermia, mycosis fungoides and skin reticulosis A uniform pattern of several fundamental abnormalities was evaluated (1) excessive monocytopoietic hyperproliferation focusing on promonocytes which exhibited lymphocyte size (2) premature monocyte release from the bone marrow into the blood (3) marked increase of pyknotic monocytes circulating and (4) frequent occurrence of blood leukocytes which were characterized by large round nuclei, absence of NaF sensitive esterase and high DNA synthesis activity There was some evidence indicating that these cells represent promonocytes with low degree of cytoplasmatic differentiation This pattern of monocytopoietic abnormalities was uniformly present in virtually all patients It did not correlate with the clinical diagnosis or with the extent of skin lesions

Key Words Disorders of monocytopoiesis Erythrodermia Kinetics of monocytopoiesis Monocytes Mycosis fungoides Skin reticulosis

Erythrodermia, mycosis fungoides and skin reticulosis share several characteristics in common The clinical course resembling that of malignant diseases, interchanges in symptomatology, and more or less widespread autonomous cellular skin infiltrations which are predominantly composed of histocytes It is well established today that histiocytes of normal skin, as well as macrophages appearing at the site of inflammatory reactions, arise from blood monocytes The monocytes, in turn, are regenerated from promonocytes in the bone marrow [4, 6, 13, 16, 19,

[1] Research supported by Mueller Fahnenberg Donation and Swiss Cancer League grant No FOR 034 AK 73 (1)

20]. Therefore, in order to elucidate the nature of the above-mentioned diseases whose pathogenesis is not well defined, a detailed study of monocytopoiesis and blood monocytes was carried out.

Materials and Methods

Subjects. Consent for the investigations was obtained from 9 patients, mean age 59 years (range 39–70 years). The disease involved the whole integument in 7 patients, 3 cases with erythrodermia, 3 mycosis fungoides, and 1 reticulosis of the skin. In another patient with skin reticulosis skin affections were localized in the back and left upper arm. Still a further case of skin reticulosis was studied during partial remission following radiotherapy. Skin lesions were restricted to the right forehead and upper part of the nose. The patients examined had neither severe concomitant diseases nor signs of infection. Treatment with corticosteroids or cytotoxic drugs was discontinued at least 3 weeks prior to the study.

Examination of blood leukocytes. Routine cell counts were performed using a Coulter Counter. 400 white cell differential counts were made on May-Grünwald-Giemsa stained smears. Aliquots of 4 ml venous blood were mixed with Na_2-EDTA plasmagel (1.107 g Na_2EDTA+1.4 g NaCl in 100 ml distilled water+100 ml plasmagel) and 10 μCi ^{3}H-TDR (specific activity 5 Ci/mM). The mixture was allowed to sediment for 30 min. Subsequently, the supernatant was pipetted off and spun at 160 g for 5 min. Concentrated leukocyte smears suited for microscopic evaluation were prepared from the sediment. These smears were submitted to May-Grünwald staining, demonstration of naphthol AS-D-chloroacetate esterase [7], NaF resistant and NaF sensitive naphthol AS-D acetate esterase [14, 15].

500 polychrome stained monocytes were arbitrarily differentiated into three groups according to nuclear morphology: (1) monocytes with large round or oval nuclei, (2) with reniform or slightly folded (intermediate forms) and (3) distinctly folded nuclei.

Indices of reaction intensity for naphthol AS-D-chloroacetate esterase were established for each of the three types of blood monocytes by arbitrarily classifying 500 cells into four groups according to reaction activity: 0 = negative, 1 = slightly positive, 2 = medium positive and 3 = strongly positive. The activity index was calculated as follows: sum of the products of cell number within a group times group number related to 100 monocytes.

Smears exhibiting the combined reactions of NaF-sensitive and NaF-resistant naphthol AS-D acetate esterase were submitted to dipping film autoradiography (Ilford L_4, 13 days exposure) and consecutive staining of nuclei with hemalaune (15 min) through the film layer. Hemalaune adsorbed by the film was carefully leached out by incubation in lukewarm water. In these preparations 2,000 cells demonstrating positive enzyme reaction were scanned and divided with regard to nuclear morphology, enzyme reaction intensity (1 = slightly positive, 2 = medium positive, 3 = strongly positive) and ^{3}H-TDR incorporation *in vitro* (nuclei overlaid by more than 4 grains were judged as being labeled). Enzyme activity indices were calculated as described above.

Examination of monocytopoiesis is reported in detail elsewhere [11] therefore only the principle of the study and important selected data are considered in this present paper Bone marrow spicules washed free of contaminating blood were incubated in autologous serum, containing 12 μCi ³H TDR, for 30 min at 37 °C. Subsequently bone marrow smears were prepared. Some of the preparations were submitted to polychrome staining for establishing bone marrow differential count (1 000 nucleated cells were scanned) Some smears were used to demonstrate the monocytic series by use of combined reactions of NaF-sensitive and NaF resistant naphthol AS-D-acetate esterase [14, 15] The relative number of promonocytes was determined by recording 3 000 nucleated bone marrow cells Some smears, demonstrating this enzyme reaction, were submitted to autoradiographic processing using Ilford L_4 dipping emulsion and 13 days exposure Following nucleus staining with hemalaune ³H TDR labeling indices (LI) were determined by recording 1 000 promonocytes Nuclei overlaid by more than 4 grains were judged as being labeled (mean background 1 1-2.3 grains per nucleus)

According to nuclear morphology promonocytes were divided somewhat arbitrarily into four groups type I = lymphocyte like cells bearing small round or oval nuclei type II = cells of myelocyte size with large round or oval nuclei type III = cells of myelocyte size with large reniform or slightly folded nuclei, and type IV = cells of myelocyte size with distinctly folded nuclei

The total marrow pool of hematopoietic cells and pools of different monocyte precursor types were calculated on the basis of data given by DONOHUE *et al* [2] Monocyte birth rate (BR) by mitotic division within the intramedullary proliferation pool of monocytopoiesis was calculated according to the formula reported by CRONKITE *et al* [1] BR = cell number within a compartment times LI divided by DNA synthesis time (T_s = 10 h [11])

Results

The data evaluated in the patients exhibited a rather uniform pattern of abnormalities showing no specific correlation to the clinical diagnosis or to the extent of the skin lesions Therefore, it seemed justified to treat the data statistically as samples of one population

Blood monocytes Moderate and marked monocytosis, ranging from 585 to 1,275 monocytes/μl, was present in 6 of 9 patients Monocytosis was caused mainly by the increase of round or oval nucleated monocytes reaching 4 times the normal values (table I) The reaction of naphthol AS D-chloroacetate esterase was intensified whereas the reaction of NaF sensitive naphthol AS-D acetate esterase was slightly depressed ³H TDR labeling indices of cells demonstrating positive reaction of NaF sensitive esterase were normal (table I) No atypical leukocytes were detected in May-Grunwald stained blood films or concentrated leukocyte smears

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Materials and Methods

Subjects Consent for the investigations was obtained from 9 patients, mean age 59 years (range 39–70 years) The disease involved the whole integument in 7 patients 3 cases with erythrodermia 3 mycosis fungoides and 1 reticulosis of the skin In another patient with skin reticulosis skin affections were localized in the back and left upper arm Still a further case of skin reticulosis was studied during partial remission following radiotherapy Skin lesions were restricted to the right forehead and upper part of the nose The patients examined had neither severe concomitant diseases nor signs of infection Treatment with corticosteroids or cytotoxic drugs was discontinued at least 3 weeks prior to the study

Examination of blood leukocytes Routine cell counts were performed using a Coulter Counter 400 white cell differential counts were made on May Grünwald Giemsa stained smears Aliquots of 4 ml venous blood were mixed with Na_2 EDTA plasmagel (1 107 g Na_2EDTA+1 4 g NaCl in 100 ml distilled water+100 ml plasmagel) and 10 μCi ^{3}H TDR (specific activity 5 Ci/mM) The mixture was allowed to sediment for 30 min Subsequently the supernatant was pipetted off and spun at 160 g for 5 min Concentrated leukocyte smears suited for microscopic evaluation were prepared from the sediment These smears were submitted to May Grünwald staining demonstration of naphthol AS D chloroacetate esterase [7] NaF resistant and NaF sensitive naphthol AS D acetate esterase [14 15]

500 polychrome stained monocytes were arbitrarily differentiated into three groups according to nuclear morphology (1) monocytes with large round or oval nuclei (2) with reniform or slightly folded (intermediate forms) and (3) distinctly folded nuclei

Indices of reaction intensity for naphthol AS-D-chloroacetate esterase were established for each of the three types of blood monocytes by arbitrarily classifying 500 cells into four groups according to reaction activity 0 = negative 1 = slightly positive 2 = medium positive and 3 = strongly positive The activity index was calculated as follows sum of the products of cell number within a group times group number related to 100 monocytes

Smears exhibiting the combined reactions of NaF sensitive and NaF resistant naphthol AS-D acetate esterase were submitted to dipping film autoradiography (Ilford L_4 13 days exposure) and consecutive staining of nuclei with hemalaune (15 min) through the film layer Hemalaune adsorbed by the film was carefully leached out by incubation in lukewarm water In these preparations 2 000 cells demonstrating positive enzyme reaction were scanned and divided with regard to nuclear morphology enzyme reaction intensity (1 = slightly positive, 2 = medium positive 3 = strongly positive) and ^{3}H TDR incorporation *in vitro* (nuclei overlaid by more than 4 grains were judged as being labeled) Enzyme activity indices were calculated as described above

Examination of monocytopoiesis is reported in detail elsewhere [11], therefore, only the principle of the study and important selected data are considered in this present paper Bone marrow spicules washed free of contaminating blood were in cubated in autologous serum, containing 12 μCi ^{3}H TDR, for 30 min at 37 °C. Subsequently, bone marrow smears were prepared. Some of the preparations were submitted to polychrome staining for establishing bone marrow differential count (1 000 nucleated cells were scanned) Some smears were used to demonstrate the monocytic series by use of combined reactions of NaF sensitive and NaF resistant naphthol AS-D-acetate esterase [14 15] The relative number of promonocytes was determined by recording 3 000 nucleated bone marrow cells Some smears demon strating this enzyme reaction, were submitted to autoradiographic processing using Ilford L_4 dipping emulsion and 13 days exposure Following nucleus staining with hemalaune, ^{3}H TDR labeling indices (LI) were determined by recording 1 000 promonocytes Nuclei overlaid by more than 4 grains were judged as being labeled (mean background 1 1–2 3 grains per nucleus)

According to nuclear morphology, promonocytes were divided somewhat arbi trarily into four groups type I = lymphocyte like cells bearing small round or oval nuclei type II = cells of myelocyte size with large round or oval nuclei type III = cells of myelocyte size with large reniform or slightly folded nuclei and type IV = cells of myelocyte size with distinctly folded nuclei

The total marrow pool of hematopoietic cells and pools of different monocyte precursor types were calculated on the basis of data given by DONOHUE *et al* [2] Monocyte birth rate (BR) by mitotic division within the intramedullary prolifera tion pool of monocytopoiesis was calculated according to the formula reported by CRONKITE *et al* [1] BR = cell number within a compartment times LI divided by DNA synthesis time (T_s = 10 h [11])

Results

The data evaluated in the patients exhibited a rather uniform pattern of abnormalities showing no specific correlation to the clinical diagnosis or to the extent of the skin lesions Therefore, it seemed justified to treat the data statistically as samples of one population

Blood monocytes Moderate and marked monocytosis, ranging from 585 to 1,275 monocytes/μl, was present in 6 of 9 patients Monocytosis was caused mainly by the increase of round or oval nucleated monocytes reaching 4 times the normal values (table I) The reaction of naphthol-AS D-chloroacetate esterase was intensified, whereas the reaction of NaF-sensitive naphthol-AS D acetate esterase was slightly depressed ^{3}H-TDR labeling indices of cells demonstrating positive reaction of NaF sensitive esterase were normal (table I) No atypical leukocytes were detected in May-Grunwald stained blood films or concentrated leukocyte smears

Table I Counts and functional characteristics of blood monocytes, mean, SD (in parentheses) of 9 patients (P) and 10 healthy individuals (N)

	Total		Nucleus morphology							
			round		intermediate		folded		pyknotic	
	P	N	P	N	P	N	P	N	P	N
Blood count/µl	668 (278)	370 (110)	127 (65)	30 (10)	196 (112)	120 (45)	334 (160)	220 (85)	5.6 (3.7)	0.6 (0.6)
Activity index of naphthol-AS-D-chloroacetate esterase	99 (24)	42 (11)	105 (34)	128 (58)	78 (14)	53 (21)	105 (37)	26 (11)	–	–
Activity index of NaF-sensitive naphthol-AS-D-acetate esterase	–	–	150 (38)	205 (10)	216 (28)	230 (8)	199 (23)	218 (5)	–	–
^{3}H-TDR labeling index, %[1]	0.2	0.2	–	–	–	–	–	–	–	–

[1] Cells demonstrating positive reaction of NaF-sensitive naphthol-AS-D-acetate esterase

Table II Blood count and ^{3}H TDR incorporation *in vitro* of leukocytes demonstrating large monocyte-like nuclei, however, negative reaction of NaF sensitive naphthol AS-D-acetate esterase, mean, SD (in parentheses)

	Patients					Healthy individuals				
	n	total	round	inter mediate	folded	n	total	round	inter-mediate	folded
%	9	100	85.2	7.4	7.4	5	100	73.1	15.4	11.5
Blood count/µl	9	35.1 (5.2–127.5)	29.9 (4.7–108.3)	2.6 (0.2–9.3)	2.6 (0.2–9.8)	5	2.6 (0–4.7)	1.9 (0–4.2)	0.4 (0–1.2)	0.3 (0–0.7)
^{3}H TDR labeling index %	9	25.2	27.9	12.1	3.4	8	20.1	23.6	8.8	–

Table III Naphthol AS D-chloroacetate esterase reaction intensity and ^{3}H TDR incorporation *in vitro* of large blood leukocytes (mean cell diameter ~20 µm) bearing round or oval nuclei (analysis of 1,200 cells from 4 patients)

	Degree of reaction intensity			
	0	1	2	3
Cells, %	6.3	1.4	7.2	85 1
^{3}H TDR labeling index, %	92	53	8	2

Pyknotic blood monocytes In concentrated leukocyte smears a small number of cells were observed exhibiting intense and homogenous reaction of NaF-sensitive esterase and pyknotic nuclei These generally appeared as one or two structureless chromatin droplets without connecting chromatin bridges The mean count of these cells, which were considered as pyknotic monocytes, reached 5 6/µl (table I)

To our knowledge this cell type has never been described before Therefore, blood samples of 10 healthy individuals were examined Pyknotic monocytes were observed, too, however mean counts reached only 0 6/µl

Large leukocytes with monocyte-like nuclei but with the absence of NaF-sensitive esterase reaction were observed in concentrated leukocyte smears of the patients as well as in the normal individuals The counts differed remarkably, yielding mean values of 35 cells/µl in patients and 3 cells/µl in controls The largest cell diameter averaged 21 6 µm (range 18 7–26 7 µm), mean smallest cell diameter averaged 19 2 µm (range 16 0–22 7 µm) The mean frequency distribution of round or oval nucleated cells, cells with reniform and folded nuclei, was 85 2 7 4 7 4% (table II) ^{3}H-TDR labeling indices of round nucleated forms reached remarkably high values, approximating 28% In contrast, cells with reniform and folded nuclei yielded 12 and 3%, respectively

In an attempt to further characterize these enigmatic large leukocytes naphthol AS D-chloroacetate esterase and ^{3}H-TDR incorporation was evaluated simultaneously in individual cells Table III summarizes the data obtained by analyzing 1,200 large leukocytes with cell diameters of approximately 20 µm and large round or oval nuclei taken from 4 patients 85% of these cells showed intense enzyme reaction, however, low DNA synthesis activity The remaining cell fraction demonstrated a sharp increase of DNA synthesis activity paralleling a decrease of en-

Table IV Monocytopoiesis of the patients compared with results of healthy individuals (pool n=9, LI and BR n=7)

Monocyte precursors		Patients x̄ (range)	Normal x̄ (range)	x̄ patients / x̄ normal
Type I	pool	261 (101–637)	30 (18–54)	8 7
	LI	37 (25–54)	7 (2–17)	5.2
	BR	8 7 (3 8–19.5)	0 15 (0 05–0 39)	58 0
Type II	pool	272 (192–361)	176 5 (143–219)	1.5
	LI	58 (49–71)	10 (8–14)	6 0
	BR	15 5 (9 8–19 1)	1 67 (1.3–2.3)	9.3
Type III	pool	149 (61–324)	279 (207–432)	0 5
	LI	72 (48–90)	10 (7–13)	7 1
	BR	10.2 (3 6–20.2)	2.98 (2.1–4 8)	3 4
Type IV	pool	177 (73–286)	79 (56–137)	2.2
	LI	70 (51–81)	25 (22–28)	2.8
	BR	11.9 (6 0–18 8)	2.04 (1 4–3 8)	5.8
Total	pool	858 (612–1,346)	584 (439–775)	1.5
	LI	55 (44–71)	12 (9–14)	4 6
	BR	46 4 (32.0–59 1)	6 84 (5 1–9.5)	6 8

Pool = cells × 10^6/kg body weight, LI = ^{3}H TDR labeling index *in vitro* (%), BR = cell birth rate (cells × 10^6/kg/h)

zyme activity Extremely high ^{3}H-TDR labeling indices yielding 92% were observed in enzyme negative cells

Bone marrow examinations The myeloid-erythroid ratio shifted slightly in favor of the erythroid series in all but one patient. Three cases demonstrated moderate eosinophilia. Judging by polychrome staining, there was a substantial increase of reticulum cells in all patients The relative numbers of these cells averaged 5 9% and ranged between 3 8 and 9 8% (normal mean 0 4%, range 0 1–0 9%)

Pools of promonocytes The total promonocyte pool was regularly increased and exceeded the normal values by a mean factor of 1 5 (table IV) This increase was predominantly caused by an expansion of the type I precursor compartment (type I precursors = small promonocytes of lymphocyte size and round or oval nuclei)

^{3}H-TDR labeling indices of all promonocyte types were regularly elevated The average of the pooled precursors yielded 4 6 times the normal level (table IV)

Monocyte birth rate Enlarged pools, as well as a rise in ^{3}H-TDR labeling indices of promonocytes, produced a striking increase in the monocyte birth rate yielding about 8 times the normal values (table IV) Excessively high birth rates, exceeding the norm by factors of approximately 60, were evaluated in the compartment of the small lymphocyte-like type I promonocytes

Discussion

A rather uniform pattern of abnormalities concerning monocytopoiesis and blood monocytes was observed in all patients examined This pattern did not specifically correlate to the clinical diagnosis, e g erythrodermia, mycosis fungoides and skin reticulosis, or to the extent of the skin lesions The monocyte birth rate by mitotic division, within the monocytopoietic proliferation pool, was strikingly increased This increase was caused by two components the expansion of the promonocyte pool and a rise in their DNA synthesis activity These results indicate a reduction of the cell generation time The time gained by the shortening of the cell cycle allows additional mitotic cycles to be performed by the promonocytes during the passage through the proliferation pool It is of interest to note that maximum hyperproliferation was associated with the small lymphocyte-like type I promonocytes Within the compartment of these precursors, the monocyte birth rate yielded approximately 60 times the normal values

Previous studies revealed that proliferation capacity of monocytopoiesis is only partially utilized under normal conditions The proliferation reserve was predominantly associated with the type I precursor compartment [9] The rise in monocyte birth rate within this type I precursor compartment was observed equally in patients with infections and inflammation The rate of increase, however, was limited to only 20 times the normal levels

The pattern of abnormalities of blood monocytes found in the present study corresponded partially to the pattern observed in patients with infections [10, 12] According to previous studies, it is suggested that monocytosis was due to the rise in monocyte influx rate [10, 12] The increased cell influx, in turn, was correlated with premature monocyte release from the bone marrow into the blood This line of reasoning was evidenced by the following findings (1) increase of relative and absolute numbers of monocytes with round or oval nuclei, (2) elevated naph-

thol-AS-D-chloroacetate esterase activity in combination with depression of NaF-sensitive naphthol AS-D acetate esterase activity

Two unknown and undefined leukocyte forms were detected in the patients' blood Further study revealed the presence of such cells in the blood of normal individuals The ratio of cell counts normal patient averaged 1 10 One of these leukocyte forms was considered to be a pyknotic monocyte This conclusion based on two arguments (1) strongly positive reaction of NaF sensitive esterase being homogenously distributed within cytoplasma, and (2) nuclei appearing as structureless chromatin droplets These nuclei resembled those of the pyknotic neutrophils [3, 5, 17] The rise of pyknotic monocytes in the blood may either indicate impaired cell clearance or increased intravascular monocyte death rate The latter explanation is supported by the discrepancy between excessive monocytopoietic hyperproliferation on the one hand and only mild monocytosis on the other

Blood counts of a further leukocyte form, whose nature is still somewhat enigmatic, yielded an average of 35/μl in patients and 3/μl in normal subjects These cells exhibited the following characteristics (1) mean cell diameter 20 μm, (2) large round or oval nuclei, (3) complete absence of NaF-sensitive naphthol AS D-acetate esterase, (4) negative to intense reaction of naphthol-AS-D-chloroacetate esterase, and (5) high DNA synthesis activity, maximal values being present in naphthol-AS D-chloroacetate esterase negative cells These cells could not be ranked into the granulocytic or the lymphocytic series In the first instance because of the absence of the 'shift to the left' in differential count and the absence of naphthol-AS-D-chloroacetate esterase reaction in a certain cell fraction In the second instance on account of the large cell diameters The occurrence of intermediate forms between the questionable leukocytes and normal blood monocytes gives rise to the assumption that the questionable cells represent immature forms of the monocytic series It seems likely that the cells by virtue of an early stage of cytoplasmatic differentiation did not demonstrate NaF sensitive esterase activity

Implications of the findings with respect to the pathogenesis of diseases examined Erythrodermia, mycosis fungoides and skin reticulosis appear, from the clinical point of view, as strongly related entities This concept is directly supported by the present studies which reveal a uniform pattern of fundamental abnormalities of the monocytopoietic system in virtually all patients examined The fact that the abnormalities of

monocytopoiesis did not correlate with the extent of dermal lesions gives rise to an additional assumption, namely, that the skin affections merely represent more or less occasional symptoms of an autonomous disorder concerning the monocyte-macrophage system

Acknowledgement The authors wish to thank Mrs B KASTEN for her skilful technical assistance

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Acta Haemat 50 340-349 (1973)

Synthese von Hamoglobin, RNS und Proteinen in der normalen Erythropoese[1,2]

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Abstract Quantitative photometric investigations on erythroblasts show (1) the most pronounced formation of RNA and haemoglobin takes place in young erythroblasts (2) Besides the synthesis of haemoglobin in mature erythroblasts no longer capable of mitosis new formation of RNA continues (3) In the case of erythroblasts of the G_1 phase with nuclei of various sizes, a critical concentration of haemoglobin is achieved, but not exceeded (4) The stem cells are transformed during the G_1 phase predominantly into large, but occasionally into small erythroblasts as well (5) Also in normal erythropoiesis, a varying number of generation cycles takes place

Key Words
Cytophotometry
Erythropoiesis
Haemoglobin formation
Nucleic acids
RNS synthesis

Thorell [1] publizierte 1947 die ersten quantitativen Befunde uber Veranderungen des Nukleinsaure-, Protein- und Hamoglobingehalts in der Einzelzelle im Ablauf der Erythroblastenreifung Er schlug damit fur die Erythropoese erstmals eine Brucke zwischen Biochemie [12] und Zellmorphologie In der Zwischenzeit konnten durch zytophotometrische [2, 3, 8] und autoradiographische [9, 16, 17, 22] Untersuchungen direkte Angaben uber die Neubildungsraten einzelner Zellbausteine gewonnen werden Ziel der vorliegenden Arbeit ist es, gleichzeitig das Ausmass der DNS- sowie der zytoplasmatischen RNS-, Protein- und Hamoglobinvermehrung in den verschiedenen normalen Erythroblasten-

[1] Herrn Prof Dr Dr h c H E Bock zum 70 Geburtstag gewidmet

[2] Mit Unterstützung der Deutschen Forschungsgemeinschaft

generationen unter Berucksichtigung der interphasischen Kerngrossenzunahme der Erythroblasten [4] durch Bestimmung der Eigenabsorption der genannten Substanzen bei verschiedenen Wellenlangen im nahen UV-Licht zu erfassen [vergl 5]

Methodik

Blut und Knochenmarkausstriche zweier Kranker mit normaler Erythropoese bzw leichter pulmonal bedingter Polyglobulie (*Tab I*) wurden auf Quarzobjektträgern ausgestrichen und nach Lufttrocknung in wasserfreiem Glyzerin eingedeckt. Die Einstellung im UV Licht erfolgte mit Hilfe einer Bildübertragung auf einen Fernsehschirm UV Absorptionsmessungen erfolgten bei den Wellenlangen 265 280 313 405 bzw 416 nm mit dem Zeiss Zytophotometer UMSP 1 Zur Bestimmung des Hamoglobinmaximums wurden fortlaufende Absorptionsmessungen zwischen 380 und 440 nm durchgefuhrt Da die Bestimmung des unspezifischen Lichtverlustes gemessen bei 313 nm infolge der niedrigen Absorption erheblichen methodischen Fehlern unterlag entschlossen wir uns einen Mittelwert, bestimmt aus zahlreichen Einzelmessungen fur jedes Präparat den anschliessenden Computerberechnungen zugrunde zu legen

Fur die Berechnung der DNS bzw RNS und Proteinmengen wurden die von uns bereits fruher angegebenen Formeln [6] verwandt

[illegible]

bez [illegible] ben sich unter gleichzeitiger Berucksichtigung der spezifischen Extinktionsfaktoren fur Proteine unter Zugrundelegung der von CASPERSSON und SANTESSON [7] angegebenen Werte

Die Karyometrie erfolgte durch Abzeichnen der Zellfläche vom Zentrum des Bildschirms und anschliessender Planimetrie Vom Patient We. wurden 1500 Zellen von Di 1000 Zellen untersucht

Fehlerdiskussion Der Fehler setzt sich zusammen aus der Genauigkeit des eingesetzten Extinktionskoeffizienten, der Wertung der unspezifischen Lichtabsorption sowie aus dem jeweiligen Verhältnis der Extinktion 265 und 280 nm d.h fur die Proteinbestimmung von der gleichzeitig vorhandenen RNS-Konzentration und umgekehrt. Unter Zugrundelegung eines 10prozentigen methodischen Fehlers in der Bestimmung von E_{265}/E_{280} betragt fur die Berechnung der Proteinmengen bei E_{265}/E_{280} = 1 2 der maximale Fehler 32% bei 1 4 50% fur die Berechnung der DNS bzw RNS Mengen bei einem Verhaltnis E_{265} zu E_{280} 1 2 maximal 20% bei 1 4 maximal 15% Ein weiterer Fehler entsteht schliesslich dadurch dass die den Kern überlappende Zytoplasmakappe der Messung nicht isoliert zuganglich ist Da das überlappende Zytoplasmavolumen den Quotienten aus der uber dem Kern areal gemessenen Hämoglobinabsorption und der zytoplasmatischen Hamoglobinabsorption nicht überschreiten kann wurden unter Zugrundelegung dieses Wertes mit Hilfe eines weiteren Computerprogrammes alle zytoplasmatischen Daten noch

Tabelle I

	Patient	
	Di ♀	We ♂
Alter, Jahre	61	67
Diagnose	Dickdarm Ca	M Boeck, Lungenfibrose
Hb, g%	12,4	17,1
Erythrozyten, Mill/mm³	3,9	5,2
HbE, pg	32	33
Erythrozytendurchmesser, µm	7,5	7,2
Retikulozyten, ‰	12	2
Leukozyten/mm³	11 900	7 600
Thrombozyten/mm³	260 000	244 000
Diff Blutbild, %		
Stab	7	2,5
Seg	66	62,5
Eos	0	3,5
Baso	0	0
Lympho	22	25
Mono	5	6,5
Sternalpunktat		
Zellzahl/mm³	84 000	140 000
Rote/100 weisse Zellen	20,5/100	38/100
Erythrop Reif z.	175	183
Sideroblasten, %	35	–
Serumeisen, µg%	79	68

ein zweites Mal berechnet. Da die Ergebnisse nicht wesentlich differieren, beziehen sich die im folgenden gemachten Angaben auf Werte, die die Kernüberlappung nicht berücksichtigen. Da die Zelldicke im Einzelfall nicht bekannt ist, können Angaben der Konzentration nur auf Menge pro µm² Zellfläche angegeben werden. Der Vergleich dieser Werte ist dennoch möglich, da die Zelldicken im Ausstrichpräparat des Knochenmarks untereinander keine wesentlichen Schwankungen aufweisen [4].

Um alle genannten Daten gleichzeitig an derselben Zelle gewinnen zu können, musste auch auf eine Vorbehandlung mit Ribonuklease zur exakten Bestimmung des Kern-DNS-Gehaltes verzichtet werden. Jeweils wurden deshalb Kern-DNS+RNS-Gehalt gemeinsam bestimmt. Der Anteil der RNS dürfte nach SANDRITTER *et al.* [6] bei andern Blutzellen 10% betragen. Da der DNS-Gehalt des Zellkerns nur zur Zuordnung der Zelle in eine bestimmte Zyklusphase verwandt wurde, dürfte diesem Fehler keine wesentliche Bedeutung zukommen.

Ergebnisse

In den beiden untersuchten Sternalmarkpunktaten wies die Mehrzahl aller Erythroblasten einen Kern DNS (+RNS) Gehalt um 7×10^{12} g auf An Hand des unterschiedlichen Kern DNS-Gehaltes wurden die zytoplasmatischen Hamoglobin-RNS- und Proteinbefunde der G_0- bzw G_1-Phase (max. $10{,}0 \times 10^{12}$g) der fruhen und mittleren S-Phase (bis 14×10^{12}g) sowie der spaten S- und G_2-Phase zugeordnet Die Befunde wurden dann der Kerngrosse als einem moglichen Mass der Zellreifung gegenuber gestellt

Hamoglobin Grosse G_0- bzw G_1-Phase Erythroblasten lassen haufig eine sichere Hamoglobinabsorption noch vermissen In den Erythroblasten aller Zyklusphasen steigt der Hamoglobingehalt pro Flacheneinheit (Abb 1) mit abnehmender Zellkerngrosse an Die höchste Hamoglobinmenge pro Flacheneinheit wird in den kleinsten G_0- bzw G_1-Phase Erythroblasten erreicht, wahrend in G_2 Phase-Erythroblasten unabhangig von der Kerngrosse eine bestimmte Hamoglobinkonzentration nicht uberschritten wird (Abb 1 unten) Die Hamoglobinzunahme in grossen G_0- bzw G_1-Phase-Erythroblasten uber S-Phase zu G_2-Phase-Erythroblasten wird noch deutlicher, wenn man den Gesamthamoglobingehalt betrachtet

Gesamtzellproteine Proteingehalt pro Flacheneinheit und Gesamtproteingehalt verhalten sich in den verschiedenen Kerngrossenklassen und Zyklusphasen ähnlich wie das Hamoglobin Die besonders niedrige Gesamtproteinkonzentration in den jungen bzw grossen Erythroblasten weist auf eine niedrige Trockengewichtskonzentration in den jungen Zellen hin

Zytoplasmatische RNS In grossen Zellen nimmt der zytoplasmatische RNS Gehalt von der G_1- über die S-Phase zur G_2 Phase deutlich zu (Abb 2) Dies kommt insbesondere dann zur Darstellung, wenn man den RNS-Gehalt nicht der Kerngrosse sondern dem Kern-DNS-Gehalt in verschiedenen Erythroblastengrössenklassen gegenuberstellt Die RNS-Neubildung nimmt während der Interphase mit zunehmender Ausreifung (d h kleinerem Zellkern) immer mehr ab Als Folge der Halbierung des RNS-Gehaltes nach jeder Zellteilung besteht eine signifikante ($p<0{,}05$) Korrelation zwischen zytoplasmatischem RNS-Gehalt und abnehmender Kerngrösse

Der Quotient RNS zur Hamoglobinkonzentration sinkt mit abnehmender Erythroblastengrosse im Ablauf des Zellzyklus von G_1 uber S

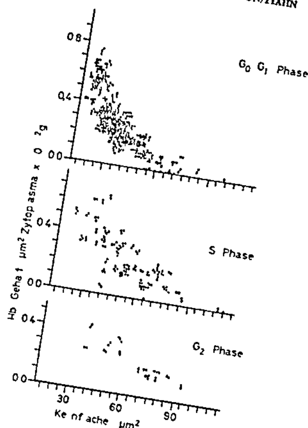


Abb. 1. Hämoglobingehalt/µm² bei verschieden grossen Erythroblasten auf Grund des Kern-DNS- (+RNS-) Gehaltes in G_0/G_1-, S-, G_2-Phase-Zellen getrennt. Patientin Dr., 61 Jahre, normale Erythropoese.

nach G_2. Das bedeutet, dass in den jüngsten, d.h. grössten Erythroblasten eine starke interphasische RNS-Neubildung und Einstrom ins Zytoplasma stattfindet, während mit zunehmender Zellreifung (kleinerer Erythroblastenkern) die Hämoglobinsynthese immer mehr in den Vordergrund tritt. In den reifen Erythroblasten mit pyknotischem Zellkern kommt es aber neben der Hämoglobinsynthese noch zu einer Zunahme des zytoplasmatischen RNS-Gehaltes. Das zeigt eine positive Korrelation ($p<0{,}05$) von Hämoglobin und RNS-Gehalt in der herausgegriffenen Gruppe mit niedrigem Kern-DNS- und RNS-Gehalt (6,3 bis $6{,}7 \times 10^{-12}$ g).

Bei dem Vergleich der Befunde an den beiden Sternalpunktaten zeigt sich, dass der höchste Hämoglobingehalt pro Flächeneinheit in den

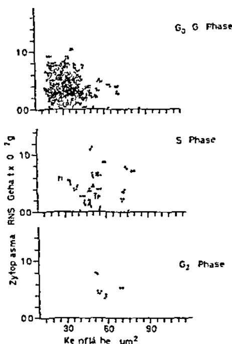


Abb 2 Zytoplasma RNS-Gehalt in verschieden grossen Erythroblasten auf grund des Kern DNS(+RNS)-Gehaltes in G_0/G_1 S- G_2 Phase Zellen getrennt. Patientin Di

kleinsten Erythroblasten bei Di hoher als bei dem Patienten We mit pulmonaler Polyglobulie ist. Umgekehrt ist bei gleichem Verhalten im Ablauf der Interphase und mit zunehmender Zellreifung der absolute RNS-Gehalt bei We grosser als bei Di

Umschriebene Zellhaufungen bei unterschiedlichen Substanzkonzentrationen als Hinweiszeichen fur verschiedene Erythroblastengenerationen konnten wir nicht nachweisen Ursache hierfur durften die fliessen den Übergange zwischen den fur die vergleichenden Untersuchungen willkurlich abgegrenzten 3 Phasen des Generationszyklus entsprechend der kontinuierlichen DNS-Zunahme von G_0 bzw G_1 nach S und G_2 sein

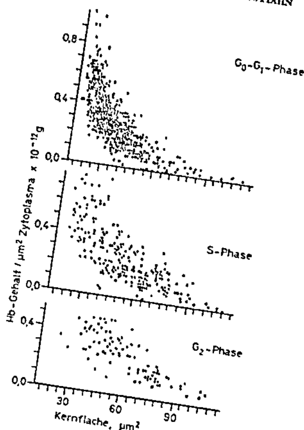


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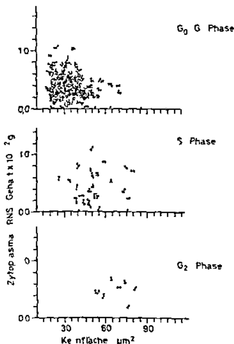


Abb 2 Zytoplasma RNS Gehalt in verschieden grossen Erythroblasten auf grund des Kern DNS(+RNS) Gehaltes in G_0/G_1 S- G_2 Phase Zellen getrennt Patientin Di

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Diskussion

Zellen mit kaum nachweisbarer Hamoglobinabsorption befinden sich aufgrund des Kern-DNS Gehaltes in G_0- bzw G_1-Phase Der vollige Mangel an Hamoglobin in diesen Zellen lasst sich am besten dadurch erklaren, dass die durch Erythropoietin induzierte Umwandlung differenzierter Stammzellen [11, 13] in morphologisch erfassbare rote Vorstufen in der G_1-Phase erfolgt. Da nur wenige grosse Erythroblasten in der G_1-Phase vorliegen, muss man annehmen, dass mit der Umwandlung zu roten Vorstufen die Zellen bereit zum Eintritt in den Zyklus sind

Neben grossen G_1- Erythroblasten ohne nachweisbare Hamoglobinmengen finden sich auch einzelne kleinere Erythroblasten bis nahezu 55 μm^2 (Di) bzw 30 μm^2 (We) Kernflache, in denen noch nicht eindeutig Hamoglobin erfasst werden kann Da bereits im ersten Zyklus der Erythroblasten Hämoglobin gebildet wird, muss man schliessen, dass Stammzellen eine Umwandlung nicht nur in grosse, sondern vereinzelt auch in kleinere Erythroblasten erfahren

Zusammen mit der Umwandlung differenzierter Stammzellen in rote Vorstufen kommt es zu einer ausgepragten zytoplasmatischen RNS-Zunahme besonders in den jungen, d h grossen Erythroblasten Die Bildung DNS-abhangiger RNS [18] erfolgt unter Erythropoietinwirkung [10], es werden stark markierte r- und t-RNS verschiedener Sedimentationskonstanten nachweisbar [15] Damit wird in der jungsten Erythroblastengeneration alle RNS gebildet, die zur Produktion der neuen Zellproteine [14, 22] erforderlich ist und uber die Aktivierung der Fermente der Hamsynthese zur gesteigerten Hamoglobinsynthese fuhrt [19] Die RNS-Neubildung erfolgt nicht nur in allen teilungsfahigen Erythroblastengenerationen, wie das auch in autoradiographischen Studien mit ^{3}H-Cytidin [9] nachgewiesen wurde, eine RNS Neubildung erfolgt vielmehr zusammen mit der weiteren Hamoglobinsynthese auch noch in den reifsten nicht mehr teilungsfahigen Erythroblasten

Die interphasische Hamoglobinzunahme scheint in den mittelgrossen Erythroblasten starker zu sein als in Proerythroblasten Diese Befunde entsprechen den biochemischen Untersuchungen an den Dichtegradienten-getrennten Fraktionen des Knochenmarks [20]

Die Proteinsynthese erfolgt in allen Erythroblastengenerationen, Befunde die sich mit den biochemischen Untersuchungen decken [21] Mit zunehmender Zellreifung nimmt dabei die Hamoglobinneubildung einen grosseren Anteil an dem Gesamtproteingehalt an [12]

In der Gruppe der G_2-Phase Erythroblasten wird eine kritische Hämoglobinkonzentration nicht überschritten Man muss daraus schliessen, dass die Zelle keinen weiteren Zyklus mehr durchläuft, sondern bei weiterer Hämoglobinneubildung nur noch ausreift Diese «kritische Hämoglobinkonzentration» wurde von STOHLMANN *et al* [16] als ein wichtiger Faktor in der Regulation der Erythropoese im Sinne eines Repressormechanismus auf die weitere DNS-Neubildung gedeutet. Die Tatsache, dass eine kritische Hämoglobinkonzentration, die nicht überschritten wird, in G_2-Phase Erythroblasten unterschiedlicher Kerngrösse anzutreffen ist, zeigt, dass sich auch in der normalen Erythropoese nicht alle Erythroblasten gleichartig verhalten und eine konstante Zahl an Reifungsstufen bis zur endgültigen Ausreifung und Kernausstossung [17] nicht durchlaufen wird

Alle an den Erythroblasten verschiedener Grössen und Zyklusphasen des Patienten We gewonnenen Ergebnisse wurden in einer vorläufigen Mitteilung [23] unter Berücksichtigung der interphasischen Grössenzunahme in einem Modell der normalen Erythropoese eingegliedert. Ausser des insgesamt höheren RNS-Gehaltes bei dem Kranken mit pulmonalbedingter Polyglobulie entsprechen die Befunde der beiden bisher untersuchten Kranken einander

Zusammenfassung

Quantitative photometrische Untersuchungen an Erythroblasten zeigen 1 Die ausgeprägteste RNS- und Hämoglobinneubildung findet in jungen Erythroblasten statt. 2 Neben der Hämoglobinsynthese geht in den reifen und nicht mehr teilungsfähigen Erythroblasten auch eine RNS-Neubildung weiter 3 Eine kritische Hämoglobinkonzentration wird von G_2 Phase-Erythroblasten verschiedener Kerngrösse erreicht, aber nicht überschritten 4 Die Stammzellumwandlung erfolgt in der G_1 Phase überwiegend in grosse vereinzelt auch kleine Erythroblasten 5 Auch in der normalen Erythropoese werden danach bis zur Erythroblastenreifung eine unterschiedliche Zahl an Generationszyklen durchlaufen

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Acta Haemat 50 350–356 (1973)

Chromosome Studies in Paroxysmal Nocturnal Haemoglobinuria[1]

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Abstract Chromosome studies have been performed in bone marrow and in venous blood cells of two patients with paroxysmal nocturnal haemoglobinuria (PNH) The frequency of aneuploidy in marrow cells was of 40 and 35%, respectively Blood lymphocytes showed aneuploidy in only one patient The data are consistent with the hypothesis that PNH can be a myeloproliferative disease, but no direct evidence has been found neither for a clonal composition of PNH nor for participation of all marrow cell lines

Key Words
Chromosome anomalies
Karyotype
Myeloproliferative disorders
Paroxysmal nocturnal haemoglobinuria

Paroxysmal nocturnal haemoglobinuria (PNH) has been recently suggested as a 'candidate' myeloproliferative disorder [1] Cytogenetic studies are relevant to this hypothesis in order to clarify if the metabolic defect(s) of red cells is (are) associated with one or more abnormal cell clones Moreover cytogenetic findings can provide supplementary data to the open question if PNH is a disorder limited to erythropoiesis or if it also affects the other marrow cell lines [2, 3] Previous chromosome investigations in PNH are quite rare [4–6], and more information is needed The present paper reports cytogenetic findings in marrow and blood cells of two patients with PNH

Patients and Methods

Patient 1 (D M) a 24 year old woman, has been studied 3 years after diagnosis All through this period she has been regularly followed up at our out patient clinic and she has been admitted at our hospital approximately every 2 months for need of blood transfusion Patient 2 (M F), a 38 year old man, has been PNH

[1] Presented in part at the 23rd Congress of the Italian Society of Haematology

Table I

	Patient 1	Patient 2
Haematocrit, %	24	25
Hb, g/100 ml	6.15	6.75
RBC/µl	1,940 000	2,800 000
Reticulocytes/µl	184 300	181 000
Neutrophilic polymorphonuclears/µl	800	2 000
Platelets/µl	105,000	168 000
Acidified serum test, % lysis	48	41
Acetylcholinesterase assay, Δ pH/h (normal range 0.5–1.0)	0.27	0.28

bearer for 8 years His need for blood transfusion was not as frequent as for patient 1.

The acidified serum test (Ham's test) and the acetylcholinesterase assay have been performed according to DACIE and LEWIS [7] Marrow cell metaphases have been obtained by a modification of TJIO and WHANG's method [8] Venous blood cell metaphases have been obtained by a modification of the method of MOORHEAD *et al* [9]

Results

Pertinent laboratory data at the time of study are reported in table I Figure 1 shows chromosome analysis of marrow cells of patient 1 Seven out of 20 metaphases have an abnormal number of chromosomes Another metaphase is pseudodiploid, lacking one chromosome from group E_{17-18}, and having an extrachromosome in group D Most of the remaining cells are hypodiploid, with chromosome loss from group E_{17-18} (3 cells), group C (3 cells) group A_2, and D (2 cells each), group F, and G (1 cell each) Figure 1 also shows karyotype analysis from venous blood cells of the same patient. Again 13 out of 20 metaphases have 46 chromosomes, but one of them is pseudodiploid, lacking one chromosome from group G, and having an extra D The remaining 7 aneuploid metaphases are spread on a basis going from 41 to 47 chromosomes Here again as in marrow cells, there is some prevalence of 45 chromosome metaphases Five cells lack one or more chromosomes from group C.

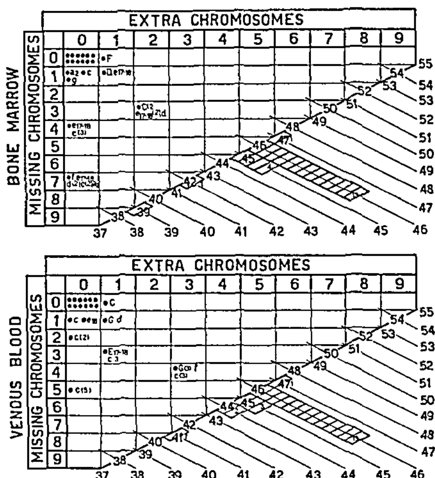


Fig 1 Patient 1 Karyotype analysis of marrow and blood cells Number of extrachromosomes per metaphase in abscissa Number of missing chromosomes per metaphase in ordinate Each metaphase is represented by a black dot whose position in the graph depends on the number of extra and missing chromosomes The metaphases with the same number of chromosomes are disposed along the same diagonal Extrachromosomes are defined by capital letters, and missing chromosomes by small letters, the letters correspond to the chromosome group affected On the right side of the graph the metaphase distribution according to chromosome number is shown by a histogram

In patient 2 (fig 2), 13 out of 20 metaphases show a normal karyotype One cell is pseudodiploid having two extra-chromosomes in group C, and one in group G, and lacking three chromosomes from group E_{17-18} Two metaphases have one extrachromosome in group C and

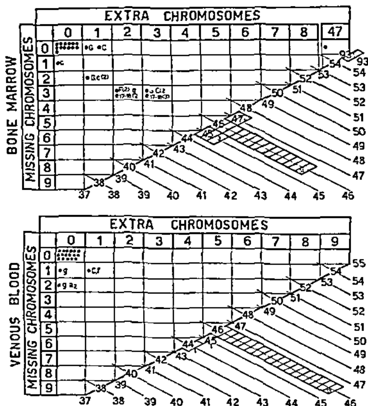


Fig 2 Patient 2 Karyotype analysis of marrow and blood cells. Interpretation as in figure 1

G, respectively Chromosome loss affects only groups C and E_{17-18}. A cell with 93 chromosomes is shown at the top right of the figure As to venous blood cell karyotype, there is a normal distribution around the mode, with one pseudodiploid cell, and two hypodiploid (44 and 45 chromosomes)

Discussion

In the two cases studied the frequency of aneuploidy is remarkably high 40% in patient 1, and 35% in patient 2 These findings partially

agree with previous reports [4–6] Pooling together the data from the two patients, 14 marrow cells out of a total of 40 examined, are aneuploid or pseudodiploid Chromosome loss affects mostly group E_{17-18} (6 cells) and group C (5 cells), but it has to be noted that contemporary loss of $E_{17\ 18}$ and of C chromosomes appears only in two cells On the contrary, most of extrachromosomes belong to groups C and G There are no E_{17-18} extrachromosomes Venous blood cell karyotype of patient 2 can be considered to be normal, while in patient 1 35% of scored metaphases are aneuploid (one of them being pseudodiploid) In this case, loss of C chromosomes is striking (5 cells), but there are no cells lacking $E_{17\ 18}$ chromosomes as in marrow

Marrow cell karyotype has been found to be normal in most haemo lytic anaemias [4, 10], while it is frequently abnormal in myeloproliferative disorders [11] Moreover, a significant aneuploidy has been found in refractory sideroblastic anaemia, another possible myeloproliferative disorder marked by a prominent defect of erythropoiesis [13 unpublished observations] Therefore, the present findings are consistent with the hypothesis that PNH is a candidate myeloproliferative disease [1, 3] Nevertheless, chromosome analyses have not provided any direct evidence for the presence of one or more abnormal cell clones It is obvious that cytogenetic observations do not rule out the possibility that PNH is a clonal disease PNH cells could have a normal or apparently normal karyotype and aneuploid or pseudodiploid cells could represent short living cell lines arising from PNH cells

The ratio erythroid metaphases granulocytic metaphases, determined on direct smears prepared from the same marrow aspirates and stained with May Grunwald Giemsa, by counting at least 100 consecutive erythroid metaphases, was 15 1 in patient 1, and 6 1 in patient 2 It is questionable to attempt any relationship between cytological distribution of mitoses in direct smears and chromosome preparations [12–15] Nevertheless, this observation provides indirect evidence that some of the erythroid precursors have to be euploid, and some have to be aneuploid, while granulocytic precursors could be all euploid or all aneuploid, or both

In conclusion, this study, while suggesting PNH as a myeloproliferative disorder, was unable to give direct evidence, neither for a clonal origin of PNH nor for participation of all marrow cell lines Keeping in mind that PNH is an acquired disease, one could speculate if a hypothetical PNH inducing factor produces chromosome abnormalities as

well as metabolic defect(s), or if a cell population characterized by an abnormal genetic (chromosome) fragility is more successful in developing PNH Whatever the truth, a pronounced genetic (chromosome) instability can explain the development and the establishment of abnormal clones [16], and then the development of acute leukemia [17–19]

The finding of a significant aneuploidy in blood lymphocytes of patient 1, who had no myeloid precursors circulating in blood, was rather surprising, also in view of previous reports [6] This observation can suggest the possibility that chromosome instability in PNH, whatever the mechanism, is a pattern of non myeloid cells too

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Acta Haemat. 50 357–363 (1973)

A New Unstable Haemoglobin: Hb Buenos Aires, β85 (F1) Phe→Ser

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Abstract A new unstable haemoglobin, Hb Buenos Aires β85 (F1) phenylalanine→serine has been found in an Argentinian family Four members were found to be heterozygous for this haemoglobin, and all suffered from a compensated haemolytic anaemia. This haemoglobin is the fifth example of an unstable variant resulting from the substitution of one of the phenylalanine residues in Hb A and underlines the importance of this large hydrophobic amino acid in maintaining the internal structure and stability of the molecule The clinical and haematological findings of the effect on carriers are discussed in terms of the nature of the mutation, and the genetic features of phenylalanine substitutions in haemoglobin are examined

Key Words
Haemoglobinopathies
Hb Buenos Aires
Unstable haemoglobins

A 17 year-old adolescent had been under observation for 5 years because of frequent attacks of jaundice associated with dark urine The attacks were more severe when he was suffering from infections As this history suggested an unstable haemoglobin as the likely cause, he was investigated for this possibility

Methods

Haematological indices were measured using routine techniques [1] Heat instability tests were carried out by diluting a haemolysate with 0 01 M tris-HCl buffer pH 6 8 and heating it for 60 min at 50 °C. Haemoglobin electrophoresis was carried out on starch gel at pH 8 6 The abnormal haemoglobin was isolated from stroma free haemolysates by precipitation with isopropanol solutions as described by CARRELL and KAY [2] The α and β-chains were separated according to the method of

Clegg *et al* [3] and preparative chromatograms carried out on the aminoethylated chains according to the methods described in Lehmann and Huntsman [4].

Case Report

The propositus (R. M.) was a 17 year-old male who was first investigated at the age of 12 years because of recurrent attacks of jaundice and pigmenturia. On questioning he admitted to a long history of attacks of lassitude associated with pallor and precipitated by infections. On clinical examination apart from jaundice and a palpable spleen (3 cm) no other abnormality was found. The haematological findings were as follows: Hb 12.6 g/100 ml, PCV 40%, red cell count $4.4 \times 10^6/mm^3$, reticulocytes 9.0%, platelets 220 000/mm^3, and WBC 7,500/mm^3. The blood film showed a few irregularly contracted cells with a moderate degree of anisocytosis, polychromasia and basophilic stippling. Bone marrow smears showed normoblastic erythroid hyperplasia. Heinz bodies were detected after sterile incubation of the patient's red cells for 48 h. On heating a haemolysate a precipitate formed within 15 min. On analysis of the haemoglobins on starch gel electrophoresis at pH 8.6 a minor fraction was detected migrating cathodially to Hb A. The Hb A_2 level was slightly raised, namely 3.9% (normal range 2.5–3.5%) and the Hb F was 1.7% (normal range up to 0.8%). The findings of other relevant investigations are given in table I.

Family history. The family was investigated over 4 generations (fig. 1). Four other members were found to be affected as judged by the presence of a haemolytic anaemia and the finding of a heat unstable haemoglobin. Also two other members were suspected to be affected on the clinical findings. The findings on this family are summarised in table II.

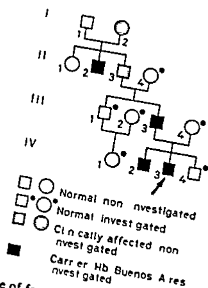


Fig. 1. Pedigree of family heterozygous for Hb Buenos Aires.

Table I Haematological and biochemical findings on patient R M.

	Patient	Normal
Haemoglobin, g/100 ml	12.6	
Haematocrit, %	40	
Red cell count/mm³	4,400,000	
Reticulocyte count, %	9 0	
Platelet count/mm³	220,000	
White blood cell count/mm³	7,500	
Serum bilirubin, mg/100 ml	1 6	0.2–0 7
Haptoglobin, mg/100 ml	absent	115
Serum iron, μg/100 ml	144	80-120
Total iron binding capacity, μg/100 ml	340	250–350
Heinz bodies, 120 min incubation	negative	negative
Heinz bodies, 48 h incubation	positive	negative
Autohaemolysis, 48 h, %	2.8	2.0
Autohaemolysis, 48 h + glucose, %	0 7	0 5
Methaemoglobin, %	2 4	0 7
Brewer's test	positive	negative
GSH, mg/100 ml	67 0	60 0–120 0
GSH reductase, U/10^{10} red cells	1 8	1.5–2 5
G-6-PD, U/10^{10} red cells	6 5	3 3–6 9
P K, U/10^{10} red cells	9 0	4 0–8 0

Table II Clinical and Haematological findings of carriers in Hb Buenos Aires

Patient[1]	Sex	Haemoglobin g/100 ml	Reticulocytes %	Clinical findings
I–2	F	–	–	pallor, jaundice, dark urine
II–2	M	13 5	4 0	jaundice, splenomegaly
II–3	M	–	–	jaundice, splenomegaly
III–3	M	13 0	6 0	jaundice, dark urine general malaise, splenectomy at 30 years of age
IV–2	M	14 0	3 5	jaundice, splenomegaly
IV–3	M	12.6	9 0	propositus, jaundice, pallor, dark urine, general malaise, splenomegaly

[1] See figure 1

Table III. Amino acid analysis of abnormal aminoethylated βTpX (83–95)

Residue	Molar ratio	Expected molar ratio
Asp	1.09	1
Thr	1.78	2
Ser	*1.87*	*1*
Glu	1.14	1
Gly	1.00	1
Ala	1.08	1
Leu	2.02	2
Phe	*nil*	*1*
His	0.90	1
Lys } AE Cys }	1.42	1 + 1

Table IV. The tryptic peptide βX (83–85)

Sequence No	83	84	85	86	87	88	89	90	91	92	93	94	95
Helical No	F1	2	3	4	5	6	7	8	9	10	11	FG1	2
Hb A	Gly	Thr	*Phe*	Ala	Thr	Leu	Ser	Glu	Leu	His	Cys	Asp	Lys
Hb Buenos Aires	Gly	Thr	*Ser*	Ala	Thr	Leu	Ser	Glu	Leu	His	Cys	Asp	Lys
				↑		↑			↑				

The peptide bonds broken with thermolysin are indicated with an arrow.

The Amino Acid Substitution

The abnormal haemoglobin was isolated by precipitation with isopropanol and dissolved in 0.1 N HCl. The globin was reprecipitated by adding the solution to 2% acid acetone at –20 °C, and the α and β chains separated. The β chain was aminoethylated, filtered on Sephadex, lyophilised and then digested with trypsin. The preparative chromatograms showed the tryptic peptide βX (residues 83–95) having lower chromatographic mobility than normal. The peptide was eluted, digested with 6 N HCl for 24 h and its amino acid composition analysed. It was found that an expected residue of phenylalanine was missing and that an extra residue of serine was present (table III). The only possible explanation for this was that the normal β85 (F1) phenylalanine was replaced by serine. However, since the size of βTpX prevents a perfect analysis, the peptides were digested with thermolysin which breaks it up at the positions indicated in table IV.

The thermolysin peptides were separated by electrophoresis at pH 6 4, and subsequent analysis on each confirmed that the phenylalanine at position 85 was replaced by serine The total haemoglobin was fingerprinted and the normal and abnormal tryptic peptides βX were eluted separately but under the same conditions, and analysed The molar ratio of the abnormal serine indicated that the abnormal haemoglobin comprised 45–50% of the total This was a hitherto undescribed mutation, and has been called Hb Buenos Aires.

Discussion

The finding of Hb Buenos Aires now brings the total number of different unstable haemoglobins to 43 [5] Unstable haemoglobins now represent the largest class of haemoglobin variants which result in disease in the heterozygous state [5] The majority has been inherited as autosomal codominant genes, but some have arisen as a new mutation They have been found in all parts of the world and in many ethnic groups After Hb Buenos Aires had been reported [6], it was later found independently in America and called Hb Bryn Mawr [7]

Phenylalanine β85 (F1) is present in the β-, γ- and δ-chains, but in the α-chain, this position is occupied by leucine The reason why this particular mutation should cause the molecule to be unstable has been explained by M F PERUTZ from the examination of the atomic model of human haemoglobin Residue β85 is an internally sited residue which acts as a spacer between the F and H helical segments If its bulky side chain is replaced by the smaller, hydrophilic side chain of serine, two things might happen First the spacing factor would be lost, and secondly, there would be a tendency for water to be attracted into the normally hydrophobic haem pocket. Both of these factors would impart instability to the haemoglobin molecule

The presence of this unstable haemoglobin causes a well compensated haemolytic anaemia and the patients have haemoglobin levels within the normal range In many of the unstable haemoglobin disorders, this has been shown to be due to the high oxygen affinity of the haemoglobin [8]

The studies of BRADLEY *et al* [7] have shown that this is the case for Hb Buenos Aires in that the whole cells and also the isolated haemoglo-

Table V. Abnormalities produced by the substitution of phenylalanine residues of Hb A

Variant	Chain	Substitution	Clinical disorder	Base change
Torino	α	CD1 Phe–Val	mild	U–G
Hammersmith	β	CD1 Phe–Ser	severe	U–C
Bucuresti	β	CD1 Phe–Leu	moderate	U–C
Christchurch	β	E15 Phe–Ser[1]	moderate	U–C
Buenos Aires	β	F1 Phe–Ser[1]	moderate	U–C

[1] Phenylalanine is not invariant in this position. In the α-chain the site is occupied by leucine.

The substitution in Hb Buenos Aires (Phe→Ser) now brings the total number of substitutions of phenylalanine in the haemoglobin molecule to 5 (table V). It is of interest that all the variants are unstable, underlining the importance of this particular large amino acid for stability of haemoglobin. It is also noteworthy that the Phe→Ser mutation produces much more instability than the mutation Phe→Val, and Phe→Leu. This can be explained by the fact that serine has a smaller side chain, and also it is the only one which has hydrophilic properties.

Amongst the unstable haemoglobins which result from a single change in the triplet base codon for an amino acid, there are 19 examples of transversion mutations and 16 examples of transitions [5]. This deviation from the expected 2:1 ratio has been pointed out before [9–11] and used as evidence that viable mutations in the haemoglobin molecule may not be occurring at random. This is supported in part by examination of phenylalanine mutations. Single-point mutations in the codons for phenylalanine, UUU, UUC, could theoretically occur 12 times: 8 transversions and 4 transitions. Therefore, it is of interest that on the 5 occasions where base changes are observed, only 1 transversion is found but 4 transitions (table V). It is unlikely that the explanation is merely that the amino acids which result from transition mutation, leucine and serine, result in more instability (thus easier detection), than those which would result from transversion mutations valine, tyrosine, cysteine, and isoleucine. However, since substitutions by the last three amino acids have not been reported, this remains speculation.

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Deutsche und Österreichische Gesellschaft für Hämatologie

Vom 21 bis 23 März 1974 finden in Wien als gemeinsame Tagung die 17 Jahrestagung der Deutschen Gesellschaft für Hämatologie und die 4 Jahrestagung der Österreichischen Gesellschaft für Hamatologie statt Präsident Prof Dr E. DEUTSCH I Medizinische Universitatsklinik Wien Sekretär Dr N HONETZ, I Medizinische Universitatsklinik Wien

Thema der Tagung Die Knochenmarkinsuffizienz (unter Ausschluss der malignen Erkrankungen)

Vorlaufiges wissenschaftliches Programm Pathologie und pathophysiologische Grundlagen 1 Die hamopoetische Stammzelle, 2. Physiologie und Pathologie der Knochenmarkmatrix 3 Anwendung von Kulturverfahren 4 Proliferationskinetik bei Panmyelopathien 5 Humorale Regulation der Hamopoese, insbesondere bei Knochenmarkinsuffizienz 6 Zytologie und Zytochemie 7 Die klinische Histologie der Knochenmarkinsuffizienz. Pathogenese und Klinik 1 Pathogenese und Klinik der Panmyelophthise 2 Isolierte aplastische Anämie 3 Agranulozytose 4 Besonderheit der Knochenmarkinsuffizienz bei Kindern 5 Enzymdefekte bei Knochenmarkinsuffizienz. 6 Toxikologie und Genetik 7 Immunologie Therapie 1 Medikamentose Beeinflussung der Knochenmarkinsuffizienz 2 Besonderheiten der Infektionstherapie 3 Probleme der Infektionsprophylaxe 4 Moglichkeiten der Behandlung der hamorrhagischen Diathesen bei Knochenmarkinsuffizienz. 5 Knochenmarktransplantation Round Table-Gesprach Die Rolle der Milz für die Entstehung der Knochenmarkinsuffizienz.

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Journées d'Hématologie radioisotopique, Montpellier

Organisées par le Laboratoire des Radioéléments de l Institut d Hématologie de Montpellier, ces Journées auront lieu le 17 et 18 avril 1974 au Centre de Transfusion Sanguine, Avenue Emile Jeanbrau, 34–Montpellier, France

Les thèmes à l ordre du jour seront les suivants 1 Les protéines plasmatiques 2 La cinétique du fer et de l érythropoïèse 3 Les tests *in vitro* à intérêt hématologique

Pour tous renseignements s adresser au Dr A WAGNER Institut d Hématologie, BP no 1213, *34010–Montpellier*, France.

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